EVIDENCE APPENDIX
EXHIBIT 10

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Multiple Dorsoventral Origins of Oligodendrocyte Generation in the Spinal Cord and Hindbrain

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Summary

Studies have indicated that oligodendrocytes in the spinal cord originate from a ventral progenitor domain defined by expression of the oligodendrocyte-determining bHLH proteins Olig1 and Olig2. Here, we provide evidence that progenitors in the dorsal spinal cord and hindbrain also produce oligodendrocytes and that the specification of these cells may result from a dorsal evasion of BMP signaling over time. Moreover, we show that the generation of ventral oligodendrocytes in the spinal cord depends on Nkx6.1 and Nkx6.2 function, while these homeodomain proteins in the anterior hindbrain instead suppress oligodendrocyte specification. The opposing roles for Nkx6 proteins in the spinal cord and hindbrain, in turn, appear to reflect that oligodendrocytes are produced by distinct ventral progenitor domains at these axial levels. Based on these findings, we propose that oligodendrocytes derive from several distinct positional origins and that the activation of Olig1/2 at different positions is controlled by distinct genetic programs.

Introduction

Neurons, oligodendrocytes, and astrocytes represent the three fundamental cell types of the vertebrate central nervous system (CNS), and the generation of these cell types at precise positions and specific time points during development is critical for the establishment of brain function. Insight has been obtained into the molecular mechanisms that control the generation of specific neuronal subtypes in space and time (Jessell, 2000; Lumsden and Krumlauf, 1996; Pattyn et al., 2003b). Oligodendrocytes and astrocytes are generated subsequent to neurogenesis (Rowitch, 2004), and less is known about the positional determination of these glial cell types during CNS development.

Oligodendrocytes are the myelinating cells of the CNS that insulate axons, while astrocytes provide structural support, regulate water balance, and maintain the blood-brain barrier (Rowitch, 2004). Glial cells originate from neural progenitors in the ventricular zone (VZ), and once specified they leave the VZ and migrate as proliferative precursors to occupy all regions of the CNS. Studies in the spinal cord suggest that oligodendrocytes are produced by a small group of ventral progenitors close to the floor plate, while astrocytes appear to be generated from more dorsally located progenitors (Hall et al., 1996; Lu et al., 2002; Pringle and Richardson, 1993; Zhou and Anderson, 2002). Functional analysis of the oligodendrocyte-determining basic-helix-loop-helix (bHLH) proteins Olig1 and Olig2 (collectively termed Olig1/2) support the idea that oligodendrocytes and astrocytes are generated from distinct progenitor domains and suggest further that oligodendrocytes and astrocytes are positionally specified (Lu et al., 2002; Zhou and Anderson, 2002; Zhou et al., 2001) according to strategies similar to those determining neuronal subtypes (Jessell, 2000).

In the spinal cord, different neurons emerge at specific dorsoventral (DV) positions in response to local Sonic hedgehog (Shh) signaling by ventral midline cells (Jessell, 2000) and bone morphogenetic proteins (BMPs) secreted from the dorsal midline of the neural tube (Lee et al., 2000; Liem et al., 1997). In ventral positions, graded Shh signaling controls patterning by regulating the regional expression of a set of homeodomain (HD)-containing transcriptional repressors (Briscoe et al., 2000; Muhr et al., 2001), thereby establishing a combinatorial code of HD protein expression, which defines five progenitor domains. Each domain, in turn, produces a distinct neuronal subtype (Jessell, 2000). Olig1/2 is induced by Shh, and its expression is confined to an individual ventral progenitor domain (termed pMN domain) that sequentially produces spinal motor neurons (sMNs) and oligodendrocytes (Rowitch, 2004). In *Olig1/2* mutant mice, pMN progenitors acquire an identity typical of more dorsal progenitors, and the loss of sMNs and oligodendrocytes in these mice is accompanied by a concomitant gain of V2 neurons and astrocytes (Lu et al., 2002; Zhou and Anderson, 2002). Apart from revealing an absolute requirement for Olig1/2 for oligodendrocyte generation, these data show that Olig1/2 also suppress astroglial fate in pMN progenitors (Zhou and Anderson, 2002), indicating that oligodendroglial and astroglial lineages are spatially separated in vivo at early developmental stages (reviewed in Rowitch, 2004).

While the data above demonstrate a restricted ventral origin of oligodendrocytes from pMN progenitors, it remains unclear if also other progenitors produce oligodendrocytes in vivo. In a series of quail-to-chick grafting experiments, Cameron-Curry and LeDouarin provided data suggesting that dorsal progenitors can produce oligodendrocytes (Cameron-Curry and Le Douarin, 1995). Similar experiments by Pringle and coworkers, however, instead argued that dorsal progenitors only generate astrocytes (Pringle et al., 1998). In vitro assays have further suggested the existence of a glial-restricted progenitor cell that can be derived from both dorsal and ventral parts of the spinal cord and give rise to oligodendrocytes and astrocytes in culture (Rao et al., 1998). Also, tripotent self-renewing stem cells that generate neurons, oligodendrocytes, and astrocytes in vitro can be isolated from most parts of the developing and adult CNS (Gage, 2000; Qian et al., 2000; Seaberg and van der Kooy, 2003), implicating that oligodendrocytes and astrocytes are derived from common precursors

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broadly distributed in the CNS. However, a recent study has questioned the presence of such tripotent stem cells in vivo, since the exposure of cells to fibroblast growth factor 2 (FGF2), the primary mitogen used to propagate stem cells, can result in a deregulated positional identity of neural progenitors in vitro (Gabay et al., 2003). The potency of progenitors isolated from a given position of the CNS may therefore reflect a quality acquired from exposure to FGF2 in vitro, rather than revealing their endogenous capacity in vivo. Thus, while it is well established that oligodendrocytes are produced by ventral progenitors, it is uncertain if also other regions of the developing CNS give rise to these cell types in vivo.

We have explored the positional specification of oligodendrocytes in the spinal cord and hindbrain and provide in vivo and in vitro evidence that, in addition to the ventral pMN domain, oligodendrocytes are generated from dorsal progenitors at both these axial levels. High concentrations of BMPs block the specification of dorsal Olig2$^-$ cells in vitro, and their generation is promoted when BMP signaling is inhibited, indicating that a progressive decrease of dorsal BMP signaling over time influences the temporal appearance of oligodendrocytes in the dorsal neural tube. In addition, we show that pMN domain-derived oligodendrocytes essentially are missing in the spinal cord of mice lacking the ventrally expressed HD proteins Nkx6.1 and Nkx6.2 (collectively termed Nkx6 proteins). We find that these HD proteins instead suppress oligodendrocyte production in the anterior hindbrain. These unanticipated opposite roles for Nkx6 proteins in the spinal cord and hindbrain, in turn, reflect that oligodendrocytes are produced by distinct ventral progenitor domains at these axial levels. Taken together, our data suggest that oligodendrocytes are generated from several distinct DV progenitor domains and that the activation of Olig1/2 at different positions is controlled by distinct genetic programs.

Results

A Dorsal Origin of Oligodendrocyte Generation in the Hindbrain

Similar to the spinal cord, oligodendrocytes in the hindbrain are generated from ventral progenitors, and their production depends on Shh signaling (Alberta et al., 2001) and Olig1/2 function (Lu et al., 2002; Zhou and Anderson, 2002). However, Olig1/2 expression in the hindbrain is also detected in the VZ at more dorsal positions (Figures 1A and 1B) (Liu et al., 2003), and the fate of these cells has not been determined. At E13.5, one dorsal *Olig1/2* expression domain spans along the axial extent of the hindbrain, with the exception of rhombomere 1 (Figure 1A). To begin to examine this population of Olig1/2$^+$ cells, we mapped their DV position in relation to the expression of HD proteins that define different DV progenitor domains. While ventral *Olig1*$^+$ cells at E13.5 were detected within the pMNv domain that expresses the HD protein *Nkx2.2* (see below), the dorsal Olig1$^+$ cells were located immediately dorsal to the expression domain of Dbx2 (Pierani et al., 1999), but within the domain of *Pax3*, *Pax7*, and *Gsh1* expression (Figures 1B–1F; data not shown). Pax3, Pax7, and Gsh1 are definitive markers of dorsal progenitor cells at this axial level (Goulding et al., 1991; Jostes et al., 1990; Sander et al., 2000; Valerius et al., 1995), suggesting that this population of Olig1/2$^+$ cells is located within the alar plate. The OLP marker *Sox10* (Kuhlbrodt et al., 1998) was expressed in a fashion similar to *Olig1* in the dorsal hindbrain (Figure 1G). As determined by immunohistochemistry, several dorsal Olig2$^+$ cells in the VZ coexpressed Gsh1 (Figure 1H) while more laterally positioned, and presumably more mature, cells coexpressed the OLP markers PDGFRα (Hall et al., 1996) and NG2 (Figures 1J and 1K) (Nishiyama et al., 1996) but not the panneuronal marker NeuN (Figure 1I) (Mullen et al., 1992). Together, these data strongly suggest that at least a subset of Olig1/2$^+$ cells in the hindbrain originates from dorsal progenitors and are consistent with the idea that these cells differentiate into oligodendrocytes.

To more extensively investigate if dorsal progenitors in the hindbrain generate oligodendrocytes, we examined the capacity of hindbrain explants to generate OLPs in vitro. In this assay, explants corresponding to the ventral and dorsal Olig1/2$^+$ domains (Figure 1X) were isolated at E10.5, approximately 2–3 days before Olig1/2$^+$ OLPs can be detected in vivo (Miller, 2002). Explants isolated from tissue intervening the ventral and dorsal Olig1/2$^+$ domains (intermediate explants; Figure 1X) were included as controls, since this Olig1/2$^-$ domain is predicted not to produce oligodendrocytes in vivo or in vitro. Explants were cultured in defined media containing platelet-derived growth factor (PDGF-AA) for various time points, and OLP generation was scored by monitoring expression of the OLP markers Olig2, PDGFRα, NG2, and the O4 antigen (Sommer and Schachner, 1981). Importantly, we did not add fibroblast growth factors (FGFs) to the culture media, since FGF2 has been shown to ventralize cultured dorsal neural progenitor cells, resulting in an arbitrary in vitro-triggered induction of Olig2 expression and oligodendrocyte differentiation (Chandran et al., 2003; Gabay et al., 2003).

In ventral and dorsal explants cultured for 6 days, an extensive number of Olig2$^+$ cells were detected, and the majority of cells coexpressed PDGFRα and NG2 (Figures 1L, 1N, 1O, and 1Q). After 8–10 days in vitro, ventral explants showed significant expression of the more mature oligodendrocyte lineage marker O4 (Figure 1T). Olig2$^+$/O4$^+$ cells were detected also in dorsal explants, albeit the number of double-positive cells was lower as compared to ventral explants (Figure 1R). Importantly, in intermediate explants cultured under identical conditions, expression of Olig2, PDGFRα, or O4 was not detected at any time point analyzed (3–10 days of culture; Figures 1M, 1P, and 1S and data not shown). Thus, progenitors isolated from a DV domain that lacks expression of Olig1/2 in vivo do not generate OLPs under these in vitro culturing conditions. Given that dorsal but not the more ventral intermediate explants generate OLPs, it is unlikely that the OLPs observed in dorsal explants represent ventrally derived OLPs that at the time of tissue isolation had migrated into dorsal positions. Moreover, the absence of OLPs in intermediate explants makes it unlikely that the generation of OLPs in dorsal explants result from a deregulated, or ventralized, potential of dorsal progenitors due to the culturing conditions. In additional support for this, we could detect expression of Pax7 (Figure 1U) but not the ventral mark-

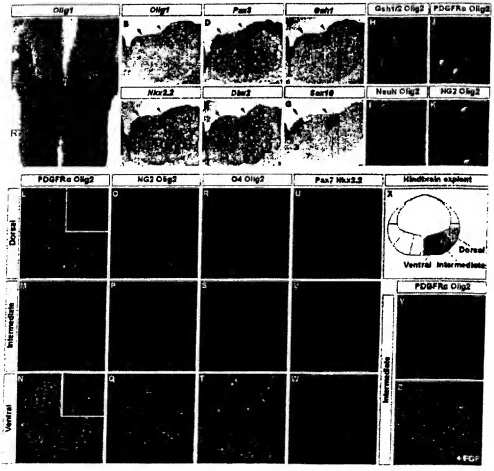

Figure 1. Dorsal Olig1/2$^+$ Progenitors in Hindbrain Give Rise to Oligodendrocytes

(A) Dorsal flat-mount view showing expression of *Olig1* in the ventral (arrow) and dorsal (arrowhead) hindbrain (HB).

(B–G) Transverse sections of rhombomere (R) 4 of the HB at E13.5 showing expression of *Olig1* (B), *Nkx2.2* (C), *Pax3* (D), *Dbx2* (E), and *Gsh1* (F). Dorsal *Olig1*$^+$ cells (ventral boundary indicated by arrowhead) are located within the domain of *Gsh1* and *Pax3*. Ventral *Olig1*$^+$ cells are detected within the *Nkx2.2*$^+$ domain (dorsal boundary indicated by arrow). *Sox10* (G) is at E12.5 expressed in a fashion similar to *Olig1* (B).

(H–K) A subset of dorsal Olig2$^+$ cells expresses Gsh1/2 (H), PDGFRα (arrows in [J]), and NG2 (arrows in [K]) but not NeuN (I).

(L–W) Ventral (vd) and dorsal (dd) but not intermediate (Ig) HB explants isolated at E10.5 generate oligodendrocytes in vitro. After 6 days in culture, Olig2$^+$/PDGFRα$^+$ cells are present in dorsal (inset in [L]) and ventral (inset in [N]) explants. After 8 days, Olig2$^+$ cells in ventral and dorsal explants express NG2 (O and Q) and O4 (R and T). No cells in intermediate explants expressed Olig2, PDGFRα, NG2, or O4 (M, P, and S). HB explants retain their dorsoventral identity, shown at 3 days in culture. Dorsal explants express Pax7 (U), while ventral express Nkx2.2 (W).

(X) Illustration of isolated dorsal, intermediate, and ventral explants.

(Y and Z) FGF2 induces Olig2 and PDGFRα expression in intermediate explants, shown after 8 days in culture.

ers Nkx2.2 or Shh (Figures 1U and 1W; data not shown) in dorsal explants cultured for 2–5 days.

It is possible that the absence of OLPs in intermediate explants could reflect that intermediate progenitors, in contrast to their more ventral or dorsal counterparts, have lost competence to generate oligodendrocytes at the time of tissue isolation. To examine this, we cultured intermediate explants in media enriched with FGF2. In contrast to explants cultured in PDGF-AA-enriched media without FGF2 (Figure 1Y), numerous Olig2$^+$/PDGFRα$^+$-expressing cells were detected in intermediate explants cultured in the presence of FGF2 (Figure 1Z). These data show that intermediate progenitors have the potential to produce OLPs and support the notion that addition of FGF2 to neural progenitor, or stem cell, cultures in vitro induces oligodendrocyte differentiation in cells not fated to generate these cells in vivo. Taken together, these data lend strong support to the idea that oligodendrocytes are derived from both ventral and dorsal Olig1/2$^+$ progenitor domains in the hindbrain.

Dorsal Progenitors in the Spinal Cord Generate Olig2-Expressing Cells and Give Rise to Oligodendrocytes in Culture

The generation of oligodendrocytes has been most extensively studied in the spinal cord (Rowitch, 2004). A

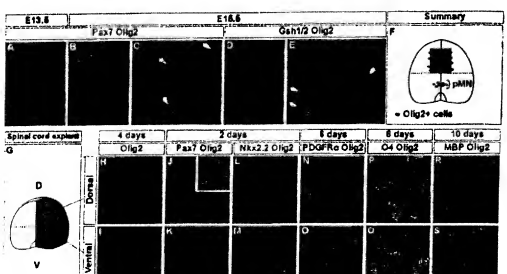

Figure 2. Dorsal Spinal Cord Progenitors Express Olig2 and Generate Oligodendrocytes In Vitro

(A–E) Transverse sections of thoracic spinal cord (SC) at E13.5 and E15.5 showing expression of Olig2 relative to Pax7 (A–C) and Gsh1/2 (D and E). Several dorsally positioned Olig2$^+$ cells coexpress Pax7 (C) and Gsh1/2 (E) at E15.5.

(F) Summary illustrating that Olig2$^+$ cells can be detected both in the ventral and dorsal ventricular zone (VZ).

(G) Illustration of the division of SC into dorsal and ventral explants.

(H–S) Whole-mount staining of dorsal and ventral explants isolated at E12.5. Cells in dorsal and ventral explants express Olig2 (H and I) and retain their dorsoventral identity after 2 days of culture; dorsal explants express Pax7 (J), and ventral explants express Nkx2.2 (M). Inset in (J) shows Olig2$^+$ cells that express Pax7. Olig2$^+$ cells in dorsal and ventral explants express PDGFRα after 6 days (N and O). O4 after 8 days (P and Q), and MBP after 10 days in vitro (R and S).

major source of oligodendrocyte production at this level is the ventral pMN domain that expresses Olig1 and Olig2 (Hall et al., 1996; Lu et al., 2002; Pringle and Richardson, 1993; Zhou and Anderson, 2002). It remains uncertain if also other progenitor domains in the spinal cord generate oligodendrocytes in vivo (Cameron-Curry and Le Douarin, 1995; Miller, 2002; Pringle et al., 1998; Richardson et al., 2000; Spassky et al., 2000). Our data suggesting a dual origin of oligodendrocytes in the hindbrain prompted us to examine the positional generation of oligodendrocytes in the spinal cord. In agreement with previous studies, the expression of Olig2 within the VZ was selectively confined to the ventral pMN domain at the peak of OLP specification at E13.5 (Figure 2A). At E15.5, migrating Olig2$^+$ OLPs were detected throughout the spinal cord (Figures 2B and 2D; data not shown). Interestingly, many Olig2$^+$ cells located within or in close proximity to the dorsal VZ coexpressed the dorsal progenitor markers Pax7 and Gsh1/2 at E15.5 (Figures 2C and 2E). Dorsal Olig2$^+$ cells located at a distance from the VZ did not express these markers (Figures 2B and 2D).

The presence of Olig2$^+$/Pax7$^+$/Gsh1/2$^+$ in the dorsal spinal cord raised the possibility that dorsal progenitors generate Olig2$^+$ oligodendrocytes at this level. Alternatively, a subset of migrating Olig2$^+$ cells generated from the pMN domain could invade the dorsal VZ and initiate expression of Pax7 and Gsh1/2 at E15. To distinguish between these possibilities, we compared the ability of isolated ventral and dorsal spinal cord explants to generate oligodendrocytes in vitro. In these experiments, ventral and dorsal explants were isolated at E10.5 or at E12 and thus prior to any dorsal migration of pMN-derived OLPs (Sussman et al., 2000). Explants were cultured in media containing PDGF-AA but not FGF2 for various time points. In these conditions, OLP differentiation was observed in both ventral and dorsal explants after 4–8 days of culture, as determined by Olig2$^+$ cells that coexpressed PDGFRα, NG2, and the O4 antigen (Figures 2N–2Q; data not shown). After 8–10 days, Olig2-expressing cells in ventral and dorsal explants had initiated expression of myelin basic protein (MBP), a marker of mature oligodendrocytes (Figures 2R and 2S) (Lemke, 1988). Similar results were obtained from ventral and dorsal spinal cord explants isolated from E10.5 and E12.5 embryos (data not shown).

We next examined the DV identity of cells in spinal cord dorsal and ventral explants. In dorsal explants isolated at E12, Olig2$^+$ cells could first be detected after 2–3 days of culture, a time point that corresponds well with the appearance of Olig2$^+$/Pax7$^+$/Gsh1/2$^+$ cells at around E15 in vivo (Figures 2J, 2L, and 2B–2E; data not shown). At these stages, Pax7 expression was observed while no expression of ventral markers Nkx2.2, Nkx6.1, or Shh could be detected (Figures 2J and 2L; data not shown). Importantly, several Olig2$^+$ cells in dorsal explants coexpressed Pax7 (Figure 2J). Similar results were obtained when dorsal explants were isolated at E10.5 (data not shown). In ventral explants, expression of Nkx2.2, Nkx6.1, and Shh but not Pax7 could be de-

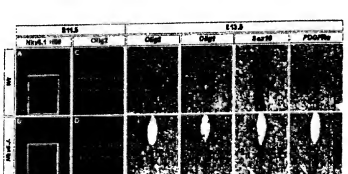

Figure 3. A Loss of Ventrally Derived Oligodendrocytes in the Spinal Cord of *Nkx6* Mutant Mice

(A–L) Transverse thoracic 8C sections of wild-type (wt) and *Nkx6* mutant mice at E11.5 and E13.5. Expression of Nkx6.1 and the MN marker Hb9 in wt (A) and *Nkx6* mutants (B) at E11.5. Box in (A) and (B) marks part of 8C shown in (C)–(L). The expression of Olig2 in the pMN domain is lost (C and D). At E13.5, expression of the OLP markers Olig2 (E and F), Olig1 (G and H), Sox10 (I and J), and *PDGFRα* (K and L) are missing in the ventral spinal cord of *Nkx6* mutant mice. Dotted lines in (C)–(L) indicate pMN domain boundaries.

tected (Figures 2K and 2M; data not shown). These data show that dorsal Pax7$^+$ progenitors generate Olig2-expressing cells and that Olig2$^+$ cells differentiate along the OLP lineage in vitro and argue against the possibility that Olig2$^+$/Pax7$^+$/Gsh1/2$^-$ detected in vivo would represent dorsally migrating cells that originate from the pMN domain. The retained DV identity of cells in dorsal explants also seems to exclude the possibility that OLPs in dorsal explants are generated in response to an in vitro-induced, albeit FGF-independent, ventralization of progenitor cell identity.

A Loss of Ventrally Derived Oligodendrocytes in the Spinal Cord of *Nkx6* Mutant Mice

To further examine the possibility of a dual ventral and dorsal origin of oligodendrocytes in the spinal cord, we analyzed mice lacking the related HD proteins Nkx6.1 and Nkx6.2. Nkx6 proteins are expressed in the ventral neural tube, including the pMN domain, and their function is necessary for the ventral expression of Olig2 and the generation of MNs in the spinal cord (Figures 3A and 3B) (Novitch et al., 2001; Vallstedt et al., 2001). The generation of oligodendrocytes in the spinal cord has not been examined in *Nkx6* mutants, but the ventral extinction of Olig2 expression raised the possibility that pMN domain-derived oligodendrocytes may be affected. In agreement with this, we could not detect any expression of *Olig1* or Olig2 in the ventral spinal cord between E11.5 and E13.5 (Figures 3D, 3F, and 3H), the time at which ventral oligodendrocytes are being specified (Hall et al., 1996). Also, while *Sox10* and *PGDFRα* could be detected in the pMN domain and/or in migrating OLPs in controls at E13.5, the expression of these OLP markers was missing in *Nkx6* mutants (Figures 3I–3L). These data show that Nkx6 proteins are required not only for the generation of spinal MNs, but also for the subsequent specification of oligodendrocytes from the pMN domain.

Oligodendrocytes Are Generated from Progenitors with a Dorsal Identity in the Spinal Cord of *Nkx6* Mutants

Olig1/2 is required for the generation of all oligodendrocytes regardless of their developmental origin in the CNS (Lu et al., 2002; Zhou and Anderson, 2002). Our data indicate that Nkx6 proteins are necessary for the generation of pMN domain-derived oligodendrocytes in the spinal cord. However, since Nkx6.1 and Nkx6.2 are expressed only in ventral progenitors, they are not predicted to affect the generation of putative dorsally derived oligodendrocytes. We therefore examined the generation of oligodendrocytes in *Nkx6* mutants at E15.5, a stage when Olig2$^+$ cells that coexpress Pax7 and Gsh1/2 can be detected in the dorsal spinal cord in wild-type embryos (Figures 2C and 2E). In controls at this stage, OLPs were evenly distributed in the spinal cord gray matter, as determined by *Olig1* expression (Figure 4C). Strikingly, numerous *Olig1*-expressing cells were observed also in *Nkx6* mutants, but in contrast to controls, essentially all *Olig1*$^+$ cells were located in the dorsal half of the spinal cord (Figure 4D). The number and distribution of Olig2$^+$ cells coexpressing Pax7 and/or Gsh1/2 in lateral positions of the dorsal VZ was similar in mutants and controls at E15 (Figures 4E–4J and 4W). These data provide strong genetic evidence that Olig1/2$^+$ cells are generated from the dorsal VZ in vivo. A few Olig1/2$^+$ cells could occasionally be detected in ventral positions at E15 (Figure 4F). Therefore, we cannot exclude the possibility that a subset of Olig1/2$^+$ cells in *Nkx6* mutants are generated also from ventral progenitors.

In control embryos at E15, a subset of Olig2$^+$ cells in the dorsal spinal cord expressed PDGFRα, while only rare Olig2$^+$/PDGFRα$^+$ cells could be detected in *Nkx6* mutants at this stage (Figures 4K and 4L). We noticed that a population of Olig2$^+$ cells that lacked the expression of PDGFRα and NG2 was present at E15.5 in wild-type mice, and this population corresponded in number to the Olig2+/PDGFRα$^-$/NG2$^-$ cells observed in mutant mice (Figure 4X; data not shown). At E18.5, Olig2$^+$ cells were evenly distributed in *Nkx6* mutants (Figure 4N), and the majority of these cells had at this stage initiated expression of PDGFRα and NG2 (Figures 4P, 4R, and 4X). Consistent with the generation of oligodendrocytes from dorsal progenitors in vitro, these data provide genetic evidence that Olig1/2$^+$ cells with a dorsal origin acquire molecular characteristics of OLPs also in vivo. Additionally, the uniform distribution of Olig1/2 cells in *Nkx6* mutants at E18.5 indicates that dorsally derived OLPs migrate into the ventral neural tube, at least in conditions when the generation of pMN domain-derived oligodendrocytes is compromised. We could not examine if OLPs in *Nkx6* mutants acquire a terminal oligodendrocyte phenotype in vivo, since *Nkx6* mutant embryos die at birth, the time when OLPs begin to terminally

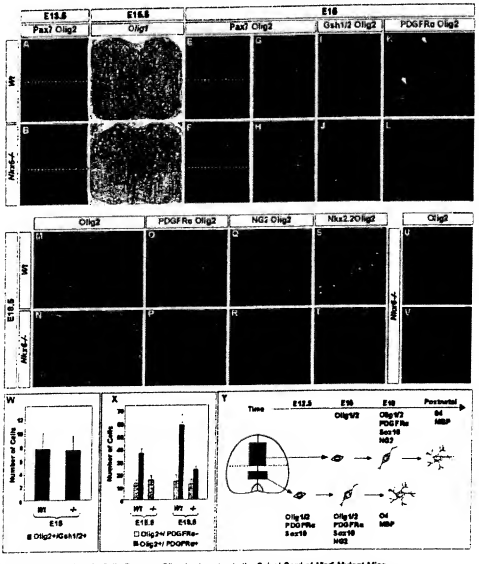

Figure 4. Dorsal Olig1/2$^+$ Cells Generate Oligodendrocytes in the Spinal Cord of *Nkx6* Mutant Mice

(A–L) Transverse thoracic SC sections of wt and *Nkx6* mutant embryos at E13.5 to E16.5. Ventral Olig2$^+$ cells are detected in controls (A) but not *Nkx6* mutants (B) at E13.5. At E16.5, Olig1 expression is detected throughout the SC of controls (C). In *Nkx6* mutants, Olig1 expression at E15.5 is detected predominantly in the dorsal SC (D). Dorsal Olig2$^+$ cells in controls and *Nkx6* mutants at E16.6 coexpress Pax7 (E–H) and Gsh1/2 (I and J). At E16.5, a small number of Olig2$^+$ cells (one to two cells per section) was also detected within, or in close proximity to, the ventral VZ in *Nkx6* mutants (F). Dotted line in (A)–(F) indicates the ventral boundary of Pax7 expression. Olig2$^+$ cells in *Nkx6* mutants do not express PDGFRα at E15.5 (L), while both Olig2$^+$/PDGFRα$^+$ cells (arrow in [K]) and Olig2$^+$/PDGFRα$^-$ cells (arrowhead in [K]) are found in controls at this stage.

(M–T) Transverse thoracic SC sections of wt and *Nkx6* mutant mice at E16.5. Olig2$^+$ cells are evenly distributed along the DV axis in wt (M) and *Nkx6* mutants (N), and the majority of cells express PDGFRα (O and P) and NG2 (Q and R). Olig2$^+$/Nkx2.2$^+$ are detected in wt (S) but not *Nkx6* mutant embryos (T).

(U and V) *Nkx6* mutant SC tissue isolated at E12.5 and cultured for 10 days generate Olig2$^+$ cells that coexpress O4 (U) and MBP (V).

(W) Quantification of Olig2$^+$/Gsh1/2$^+$ cells in wt and *Nkx6* mutants at E15.5. Counts from six to eight sections per embryo; n = 2 wt and 3 *Nkx6* mutants; mean ± SD.

(X) Quantification of Olig2$^+$/PDGFRα$^-$ and Olig2$^+$/PDGFRα$^+$ in the SC of wt and *Nkx6* mutant embryos at E16.5 and E18.5. Similar numbers of Olig2$^+$/PDGFRα$^-$ are present in wt and mutant mice at both time points. Counts from ten sections; mean ± SD.

(Y) Model indicating the differentiation profile of ventral and dorsal oligodendrocytes in the SC.

differentiate (Baumann and Pham-Dinh, 2001). Nevertheless, Olig2$^+$ cells that expressed O4 and MBP could be detected in isolated *Nkx6* mutant spinal cord tissue after culture in vitro (Figures 4U and 4V), indicating that these cells have the capacity to differentiate into mature oligodendrocytes.

Dorsal and Ventral OLP Lineages Express Distinct Molecular Properties at Prenatal Stages

Are oligodendrocytes generated from distinct positional origins molecularly and functionally equivalent? Studies of neuronal differentiation suggest that neurons with similar functional properties have certain common molecular properties, but also that functional differences among cells within a given class are associated with subtype-specific profiles of gene expression (Jessell, 2000). As compared to neuronal cell differentiation, little is known about the determination of different glial subtypes (Rowitch, 2004). To examine the molecular properties of dorsally and ventrally derived oligodendrocytes, we compared the gene expression profile of Olig2$^+$ OLPs in controls and *Nkx6* mutant mice at E18.5. In controls, a subset of Olig2$^+$/PDGFRα$^+$ cells in the spinal cord had initiated expression of Nkx2.2 (29% ± 2.5%) (Figure 4S) (Qi et al., 2001). In contrast, few if any Olig2$^+$/PDGFRα$^+$ cells in *Nkx6* mutants expressed Nkx2.2 at this stage (Figure 4T). The lack of OLPs that express Nkx2.2 in *Nkx6* mutants indicate that Nkx2.2 selectively marks OLPs with a ventral origin and that OLPs with different origins are molecularly distinct, at least at prenatal stages of spinal cord development.

Evasion of BMP Signals Influences the Timing of Olig1/2 Induction in Dorsal Progenitors

The occurrence of dorsally generated oligodendrocytes raises the issue of how these cells are specified. In the neural tube, local BMP signaling has a central role in the initial establishment of dorsal progenitor identity (Lee et al., 2000; Liem et al., 1997; Nguyen et al., 2000). BMPs expressed in the dorsal neural tube also function to suppress more ventral Shh-dependent cell fates, including MNs (Liem et al., 2000) and ventral oligodendrocytes derived from the pMN domain (Hall and Miller, 2004; Mekki-Dauriac et al., 2002). The activation of Olig1/2 expression in dorsal progenitors occurs at around E15, a stage when the neural tube has grown considerably in size. We therefore considered the possibility that dorsally generated oligodendrocytes could be sensitive to BMPs and that their late birth could reflect a decrease of BMP signaling over time. To examine this, we analyzed the number of Olig2$^+$ cells in dorsal explants that were isolated at E10.5 or E12.5 and exposed to BMP7 or the BMP antagonists Noggin and Chordin (Piccolo et al., 1996; Zimmerman et al., 1996). In dorsal explants isolated at E10.5, the generation of Olig2$^+$ cells was completely blocked when cells were exposed to 1 ng/ml of BMP7 (Figure 5G; data not shown). Interestingly, the exposure of E10.5 dorsal explants to Noggin/Chordin resulted in a 3- to 4-fold increase in the number of Olig2$^+$ cells as compared to controls (Figures 5A, 5B, and 5E). This increase did not reflect a ventralization of progenitor identity, as indicated by the in vitro detection of Olig2$^+$/Pax7$^+$ cells (Figure 5F) and a lack of any detectable induction of Nkx2.2 or Nkx6.1 expression (data not shown). Instead, the increased number of Olig2$^+$ cells in E10.5 explants exposed to Noggin/Chordin correlated with a more rapid induction of these cells as compared to controls (Figures 5H–5L). BMP7 blocked Olig2 expression also in dorsal explants isolated 2 days later at E12.5 (data not shown). In contrast to explants isolated at E10.5, however, exposure of E12.5 explants to Noggin and Chordin did not result in an increased generation of Olig2$^+$ cells (Figures 5C–5E). While these data show that dorsal progenitors are still responsive to BMPs at E12.5, the failure of BMP antagonists to promote the generation of Olig2$^+$ cells at later stages is consistent with the idea that concentration of BMPs in the dorsal neural tube decreases over time.

Oligodendrocytes Derive from Different Ventral Progenitor Domains in the Spinal Cord and Hindbrain

While Nkx6 proteins are required for Olig2 expression in the ventral spinal cord, the expression of Olig2 persists in the ventral hindbrain of *Nkx6* mutants and is even ectopically activated at anterior hindbrain levels (Pattyn et al., 2003b). The differential regulation of Olig2 in the spinal cord and hindbrain raised the possibility that the generation of ventral oligodendrocytes is regulated differently at these axial levels. In support for this, and in contrast to the spinal cord, we found an extensive ventral ectopic induction of *Olig1*, Olig2, *Sox 10*, and PGDFRα in the anterior hindbrain of *Nkx6* mutants at E12.5 as compared to controls (Figures 6H, 6J, 6L, and 6N and data not shown). These data provide direct evidence that Nkx6 proteins suppress oligodendrocyte generation in ventral positions of the anterior hindbrain. Dorsally derived OLPs in the hindbrain, however, appeared to be unaffected by the loss of Nkx6 function (Figures 6G and 6H).

How then can Nkx6 proteins mediate opposing effects on the generation of oligodendrocytes at different axial levels of the CNS? Most HD transcription factors that are involved in ventral neural patterning, including Nkx6.1 and Nkx6.2, function directly as transcriptional repressors (Muhr et al., 2001; Novitch et al., 2001; Zhou et al., 2001). These data suggest that the promotion of Olig2 expression by Nkx6 proteins in the spinal cord is indirect and possibly involves an Nkx6-mediated exclusion of a repressor of Olig genes in pMN progenitors. In the spinal cord, the Olig1/2$^+$ pMN domain is located immediately dorsal to p3 progenitors, which express Nkx2.2 and produce *Sim1*-expressing V3 neurons (Briscoe et al., 1999). Nkx2.2 is sufficient and required for the generation of V3 neurons in the spinal cord and is an established repressor of Olig2 expression at this axial level (Muhr et al., 2001; Novitch et al., 2001; Zhou et al., 2001). Unexpectedly, we found that the domain of Nkx2.2 and *Sim1* expression had expanded dorsally and encroached into the presumptive pMN domain in the spinal cord of *Nkx6* mutants at E11.5 (Figures 6A–6D). These data show a genetic requirement for Nkx6 proteins to suppress Nkx2.2 expression in the pMN domain. Further, they reveal a strong correlation between the loss of Olig2 expression, and subsequent oligodendrogenesis, and the dorsal expansion of Nkx2.2 into the pMN domain of *Nkx6* mutant spinal cord.

The observation that loss of Nkx6 proteins in the anterior hindbrain instead results in an ectopic induction of OLP markers, led us to examine the generation of OLPS at this level in more detail. In the anterior hindbrain, ventral expression of Olig2 could first be detected at E12.5 (Figures 6E, 6I, and 6K). Most, if not all, Olig2$^+$

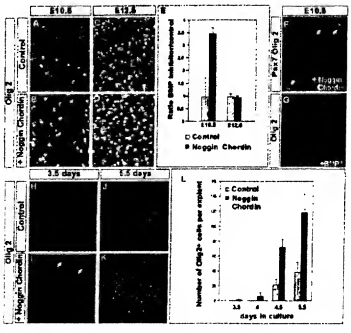

Figure 6. Decreased BMP Signaling Promotes the Generation of Dorsal Olig2⁺ Cells in Vitro

(A–D) Olig2⁺ cells in dorsal SC explants from E10.5 (A and B) and E12.5 (C and D) embryos cultured until corresponding day E18.5 in the absence (A and C) or presence (B and D) of BMP inhibitors Noggin and Chordin.

(E) Exposure of E10.5 explants to Noggin/Chordin resulted in a 3.5-fold increase in Olig2⁺ cells as compared to controls. A similar number of Olig2⁺ cells was observed in E12.5 explants cultured with or without Noggin/Chordin. Graph shows ratio of Olig2⁺ cells in Noggin/Chordin-treated explants versus controls. Eight explants analyzed per time point; mean ± SD.

(F) Explants retain their dorsal identity in the presence of Noggin/Chordin as indicated by Pax7 expression and the detection of Pax7⁺/Olig2⁻ cells (arrows in [F]).

(G) BMP7 suppresses Olig2 expression in E10.5 dorsal SC explants cultured for 8 days.

(H–K) Olig2 expression in E10.5 dorsal explants cultured for various time points in the presence or absence of Noggin/Chordin.

(L) Quantification of Olig2⁺ cells per explant. In explants exposed to Noggin/Chordin, Olig2⁺ cells could first be detected after 3.5 days of culture, as compared to 4.5 days in controls. Explants treated with Noggin/Chordin show higher numbers of Olig2⁺ cells compared to controls. Counts from six explants per time point; mean ± SD.

cells at this stage were located in lateral positions of the Nkx2.2⁺ VZ, and many cells coexpressed Nkx2.2 and Olig2 (Figures 6E and 6I). Many Olig2⁺ cells also expressed PGDFRα, indicating that they indeed are OLPs (Figure 6K). Thus, while Olig2⁺ cells in the ventral spinal cord are generated dorsal to the Nkx2.2⁺ progenitor domain, Olig1/2⁺ cells in the anterior hindbrain appear to be derived from Nkx2.2-expressing progenitors. Like spinal cord levels, the domain of Nkx2.2 expression was expanded dorsally in the anterior hindbrain of *Nkx6* mutants at E12.5 (Figures 6F and 6J; data not shown). At this axial level, however, there was a striking correlation between the expanded expression of Nkx2.2 and the ectopic induction of oligodendrocyte differentiation (Figures 6J, 6L, and 6N). These data show that the differential regulation of oligodendrocytes by Nkx6 proteins is tightly linked to the expression of Nkx2.2 and to the distinct ventral origins of oligodendrocytes in the spinal cord and anterior hindbrain (Figures 6O and 6Q).

Discussion

Previous studies have established that oligodendrocytes in the spinal cord are generated from ventral pMN progenitors in the spinal cord. In addition to a ventral origin of these cells, we here provide evidence that oligodendrocytes are produced also by progenitors in the dorsal spinal cord and hindbrain. Our study further suggests that most ventrally generated oligodendrocytes in the hindbrain are produced from Nkx2.2⁺ progenitors, rather than from more dorsally positioned pMN progenitors. Together, these data reveal multiple positional origins of oligodendrocyte specification in the spinal cord and hindbrain and provide evidence that the activation of Olig1/2 expression at different positions is regulated by distinct genetic programs.

A Dual Ventral and Dorsal Origin of Oligodendrocyte Generation in the Spinal Cord

sMNs and oligodendrocytes are generated sequentially from pMN progenitors in the ventral spinal cord, and the generation of these cells depends on the function of Olig1/2 (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). We provide several lines of evidence that, in addition to those in the pMN domain, dorsal progenitors in the spinal cord generate oligodendrocytes. First, at E15, approximately 2 days after the generation of OLPs from the pMN domain, we find that a subset of dorsal Olig2⁺ cells are located within the VZ and coexpress the established dorsal progenitor markers Pax7 and Gsh1/2 (Figures 2B–2E). Second, oligodendrocytes are produced by isolated dorsal progenitors in culture, under in vitro conditions in which cells retain their dorsal progenitor identity. Olig2⁺/Pax7⁻ cells were generated in dorsal explants isolated as early as E10.5 and, together with our analysis of *Nkx6* mutants, these experiments argue against the formal possibility that a subset of pMN domain-derived OLPs would initiate Pax7 and Gsh1 expression after migrating into the dorsal neural tube. Third, while the pMN domain and ventral oligodendrocytes are missing in the spinal cord of *Nkx6* mutants, dorsal Olig2⁺/Pax7⁺/Gsh1/2⁺ are generated on schedule and in numbers similar to those detected in controls at E15.

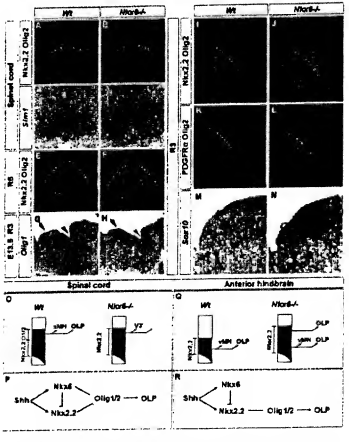

Figure 6. Different Ventral Progenitor Domains in Spinal Cord and Hindbrain Give Rise to Oligodendrocytes

(A–N) Transverse HB and SC sections of wt and *Nkx6* mutant embryos at E12.5–13.5. In the SC of wt mice, Olig2$^+$ cells are detected dorsal to the domain of Nkx2.2 expression at E12.5 (A). In *Nkx6* mutant mice, Olig2$^+$ cells are absent, and the expression of Nkx2.2 (B) and the V3 neuron marker *Sim1* (C and D) is dorsally expanded. Ventral Olig2$^+$ cells at the R6 and R3 levels of the E12.5 hindbrain are detected within, or in close proximity to, the Nkx2.2$^+$ domain (E and I). Note that several laterally positioned Nkx2.2$^+$ cells coexpress Olig2. In the *Nkx6* mutant HB, the domain of Nkx2.2 expression expands dorsally (F and J), and here the expansion is associated with an increased number and a dorsal expansion of Olig2$^+$ cells (F, J, K, and L). The generation of ectopic Olig2$^+$ cells in the ventral hindbrain of *Nkx6* mutants is accompanied by induction of OLP markers *Olig1* (G and H), PDGFRα (K and L), and *Sox10* (M and N). Dotted lines indicate dorsal boundary of wt Nkx2.2 expression. Arrow and arrowhead indicate ventral and dorsal Olig1+ domains, respectively (G and H). (O–R) Model of oligodendrocyte specification in the ventral spinal cord and hindbrain. In the ventral spinal cord and caudal-most hindbrain, somatic MNs and OLPs are sequentially generated from the Olig1/2$^+$ pMN domain located dorsal to Nkx2.2$^+$ progenitors (O). In the spinal cord, Shh induces expression of Nkx6 and Olig1/2 proteins in the pMN domain, while higher Shh concentrations induce Nkx2.2 expression in more ventral positions (P). Nkx6 proteins suppress Nkx2.2 expression in the pMN domain. The dorsal expansion of Nkx2.2 observed in *Nkx6* mutants may underlie the extinction of Olig1/2 expression in SC pMN progenitors and the ectopic generation of V3 neurons. Since Nkx6 proteins are coexpressed with Nkx2.2 in p3 progenitors, the repression of Nkx2.2 by Nkx6 is likely to require a pMN domain expressed cofactor. At anterior hindbrain levels, ventrally derived oligodendrocytes are generated from the Nkx2.2$^+$ pMNv domain that at earlier stages produce visceral MNs (C and R). Nkx6 proteins control the dorsal limit of Nkx2.2 expression also in the hindbrain. Since Olig1/2-expressing OLPs derive from the Nkx2.2$^+$ progenitor at this level, the expansion of Nkx2.2 expression in the absence of Nkx6 function therefore results in a premature and ectopic initiation of Olig1/2 expression and oligodendrocyte differentiation (Q).

We cannot formally establish that dorsal Olig2$^+$ cells acquire properties of mature oligodendrocytes in vivo, since *Nkx6* mutants die at birth and, in wild-type embryos, differentiating dorsal Olig2$^+$ cells downregulate the expression of Pax7 and Gsh1/2 and intermingle with OLPs generated from the pMN domain. Nevertheless, our data show that cells in *Nkx6* mutants differentiate along the oligodendrocyte lineage in vivo and further that mature oligodendrocytes are produced in vitro in both cultured *Nkx6* mutant tissue and wild-type dorsal spinal cord explants. Taken together, these data strongly suggest that oligodendrocytes in the spinal cord are generated from both ventral and dorsal progenitor cells. Our study also suggests that oligodendrocytes are generated from dorsal progenitors in the hindbrain, and other studies have indicated a dual origin of oligodendrocytes in the forebrain (Gorski et al., 2002; Levison and Goldman, 1993). It is possible, therefore, that the specification of oligodendrocytes from dual, or multiple, DV positions is a general characteristic of the developing CNS.

The relative contribution of the dorsal lineage of OLPs is unclear, but comparisons of the total number of Olig1/2-expressing cells in control and *Nkx6* mutant embryos imply that dorsally generated cells represent a minor fraction of the total number of OLPs at prenatal stages of development (20%–30%; data not shown). In wild-type conditions, however, this number could be lower, since dorsally specified OLPs in *Nkx6* mutants could be propagated more efficiently due to the lack of ventrally derived OLPs that are likely to compete for essential growth factors such as PDGF (Calver et al., 1998). Also, while we observe similar numbers of Olig2$^+$ cells expressing dorsal progenitor markers in *Nkx6* mutant and controls at E15, the accompanying paper by Cai et al. (2005) in this issue of *Neuron* reports an approximately 3-fold increase in the number of Olig2$^+$/Pax7$^+$ cells in *Nkx6* mutants. While the reason for this difference between our studies remains unclear, it raises the possibility that the ventral loss of Nkx6 proteins and/or ventral oligodendrocytes may have a certain influence on the specification of OLPs from dorsal progenitor cells.

How then is the specification of dorsally derived oligo-

dendrocytes regulated? Olig1/2 proteins are required for the generation of all oligodendrocytes (Lu et al., 2002; Zhou and Anderson, 2002), and a key step, therefore, must be to regulate the initiation of Olig1/2 expression in dorsal progenitor cells. Local BMP signaling from the roof plate has an essential early role in establishing dorsal neural tube identity and the patterned generation of dorsal neuronal subtypes (Helms and Johnson, 2003; Lee et al., 2000; Liem et al., 1997). Scattered Olig2$^+$/Pax7$^+$/Gsh1/2$^+$ cells can first be detected in lateral positions of the VZ at around E15 (Figures 2B–2E, 4E, and 4G). We find that BMP7 suppresses oligodendrocyte differentiation in dorsal neural tube explants in vitro, while BMP antagonists enhance generation of dorsal Olig2$^+$ cells in dorsal explants isolated at early developmental stages. Since the spinal cord has grown considerably in size by E15, these data are consistent with a model in which the timing of Olig1/2 induction in dorsal progenitors involves a progressive evasion of BMP signaling due to a limited range of action of BMP signals secreted by the roof plate.

The induction of Olig1/2 expression in the pMN domain requires Shh signaling, raising the possibility that Shh also mediates the induction of Olig1/2 expression in the dorsal neural tube. This does not appear to be the case, since data by Cai and coworkers show that dorsal oligodendrocytes are generated in the absence of Shh signal transduction in vivo (Cai et al., 2005). FGF has been shown to promote Olig gene expression and oligodendrocyte differentiation in vitro (Chandran et al., 2003; Kessaris et al., 2004), but such experiments are difficult to interpret, since the induction of oligodendrocytes in response to FGFs in vitro has also been associated with an erroneous ventralization of progenitor cell identity (Gabay et al., 2003). To overcome this issue, we exposed isolated dorsal Pax7$^+$ progenitors to an inhibitor of FGF receptor signaling, SU 5402 (Mohammadi et al., 1997), and under these conditions we observed a complete block of Olig1/2 induction and oligodendrocyte differentiation (data not shown). While additional in vivo experiments are necessary to determine the precise role for BMP and FGF signaling in this process, these data indicate that a combination of FGF signaling and a progressive decrease in BMP activity over time may underlie the late phase of oligodendrocyte specification in the dorsal half of the spinal cord.

What is then the functional rationale of producing oligodendrocytes at several DV positions? One possibility is that a single origin of oligodendrocyte specification is not sufficient to produce the number of oligodendrocytes necessary to effectively insulate all axons at a given axial level. An alternative possibility is that the production of dorsal and ventral oligodendrocytes is necessary due to the establishment of physical or molecular barriers that hamper the migration of OLPs along the DV axis. Both these alternatives, however, appear to be unlikely since OLPs, once specified, are effectively propagated in a PDGF-dependent fashion outside of the VZ (Calver et al., 1998), and both ventral and dorsal OLPs appear to be capable of freely migrating along the DV axis of the spinal cord (Rowitch, 2004) (this study). The lack of Nkx2.2-expressing OLPs in *Nkx6* mutants implies that ventrally and dorsally derived OLPs express distinct molecular properties, at least at prenatal stages of development. An intriguing possibility, therefore, is that oligodendrocytes that are generated from distinct progenitor populations acquire distinct functional properties. Analyses of Olig1/2 function directly support that patterning along the DV axis controls the spatial specification of distinct glial cells, since the loss of oligodendrocytes in the pMN domain in *Olig1/2* mutants is associated with a concomitant gain of astrocytes (Zhou and Anderson, 2002). Also, in addition to the population of myelinating oligodendrocytes, certain oligodendrocytes have been shown to establish synapses with GABAergic interneurons in the hippocampus (Lin and Bergles, 2004). Clonal analyses in the postnatal forebrain have further revealed the presence of at least two types of OLPs, one that rapidly differentiates into myelinating oligodendrocytes and another that remains undifferentiated over extensive periods of time (Zerlin et al., 2004). While the mechanism(s) that underlies these functional, or behavioral, differences among oligodendrocytes remains to be determined, it is feasible that such differences will be related to the positional specification of oligodendrocytes along the DV axis of the neural tube.

Opposing Requirements for Nkx6 Proteins for Oligodendrocyte Specification in the Ventral Spinal Cord and Hindbrain

In addition to the identification of dorsally derived Olig2$^+$ OLPs in the spinal cord and hindbrain, our study reveals a striking difference between the specification of oligodendrocytes in the ventral spinal cord and hindbrain. The activity of Nkx6 proteins is required for the generation of oligodendrocytes from the ventral progenitors in the spinal cord, while the same proteins instead suppress oligodendrocyte production at anterior hindbrain levels (Figures 6O and 6Q). Our data indicate that this differential regulation reflects that oligodendrocytes derive from distinct ventral progenitor domains at these different axial levels. In the spinal cord, the pMN domain is located immediately dorsal to the Nkx2.2$^+$ p3 progenitor domain (Briscoe et al., 1999), and Nkx2.2 is an established repressor of Olig2 expression at this level (Novitch et al., 2001). A common phenotype in the spinal cord and hindbrain of *Nkx6* mutants is that the domain of Nkx2.2 expression expands dorsally, revealing a genetic requirement for Nkx6 proteins to control the dorsal limit of Nkx2.2 expression. It is conceivable, therefore, that a primary role of Nkx6 proteins in oligodendrogenesis in the ventral spinal cord is to indirectly ensure the maintained expression of Olig1/2 through the suppression of Nkx2.2.

Our analysis indicates that, in contrast to the spinal cord, the ventral oligodendrocytes in the anterior hindbrain are generated from the Nkx2.2$^+$ pMNv domain (Figures 6I and 6K) that at preceding stages has produced visceral MNs and serotonergic projection neurons (Ericson et al., 1997; Pattyn et al., 2003a, 2003b). As a consequence, the dorsal expansion of Nkx2.2 expression in the hindbrain of *Nkx6* mutants results in an increased production of OLPs rather than a loss of these cells. It remains unclear how Nkx2.2 can promote Olig1/2 expression in the hindbrain and why Nkx2.2 and Olig1/2 are coexpressed in hindbrain progenitors but not in the mouse spinal cord. Nevertheless, the expression of

Nkx2.2 at anterior hindbrain levels shows a mutually exclusive relationship with the expression of Irx3 (Pattyn et al., 2003b), a HD protein also known to repress Olig2 expression in the spinal cord (Novitch et al., 2001). Nkx2.2 could therefore promote Olig1/2 expression, at least in part, through the exclusion of Irx3 expression in the ventral-most part of the hindbrain.

Taken together, these data establish that oligodendrocytes in the ventral spinal cord and hindbrain are generated from distinct ventral progenitor domains. Although the specification of ventral oligodendrocytes at different axial levels shows a similarity with respect to their dependence on Shh signaling (Alberta et al., 2001; Lu et al., 2002) and requirement for Olig1/2, our analysis of *Nkx6* mutant mice reveals crucial differences in the intrinsic programs that control Olig1/2 expression in the ventral spinal cord and hindbrain (Figures 6P and 6R). Considering that the loss of Nkx6 function has no significant influence on oligodendrocytes specified in dorsal positions of the spinal cord and hindbrain, it is apparent that also the upstream control of Olig1/2 expression in dorsal progenitors must be differently regulated as compared to their ventral counterparts.

Experimental Procedures

Mouse Mutants

The generation and genotyping of Nkx6.1- and Nkx6.2-deficient mice have previously been reported (Sander et al., 2000; Vallstedt et al., 2001).

Neural Tube Explant Cultures

Rhombomeres 4–6 from hindbrains of E10.5 mouse embryos (CB57) were divided into ventral, intermediate, and dorsal portions. Explants were embedded in collagen (Cohesion Technologies) and cultured as previously described (Sussman et al., 2000), with the exception that 1% FBS was replaced with 1% KCR (Invitrogen). Spinal cords from E10.5 and E12.5 mouse embryos (CB57) were divided into ventral and dorsal portions and cultured under same conditions. For assessments of FGF effects, 20 ng/ml FGF2 (Invitrogen) was added to media. Functional blocking of FGF signaling was performed by adding 25 μM SU402 (Calbiochem) to the media. For assessments of BMP effects, 1 ng/ml BMP7 (R&D Systems) was added; blocking of BMP signaling was performed by adding 1 μg/ml Chordin and 1 μg/ml Noggin (R&D Systems).

Immunohistochemistry and In Situ Hybridization Histochemistry

Immunohistochemical localization of proteins was performed as described (Briscoe et al., 2000). Antibodies used were as follows: rabbit (r), mouse (m), and guinea pig (gp) Olig2 (Novitch et al., 2001), rat PDGFRα (PharMingen), m O4, rat MBP, r NG2 (Chemicon), m Pax7, m HB9, m Shh (DSHB), r Gsh1/2 (kind gift from Martin Goulding), m NeuN (Chemicon), r Nkx6.1, r Nkx2.2 (Briscoe et al., 2000). In situ hybridization histochemistry on sections or as whole mounts was performed (Schaeren-Wiemers and Gerfin-Moser, 1993) using mouse *Olig1*, *Olig2*, *Sox10*, *Dbx2*, *Nkx2.2*, *Pax3*, *Gsh1*, and *PDGFRα* probes.

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EVIDENCE APPENDIX
EXHIBIT 11

Cai et al., "Generation of Oligodendrocyte Precursor Cells From Mouse Dorsal Spinal Cord Independent of *Nkx6* Regulation and *Shh* Signaling," *Neuron* 45:41-53 (2005)

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Generation of Oligodendrocyte Precursor Cells from Mouse Dorsal Spinal Cord Independent of *Nkx6* Regulation and *Shh* Signaling

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Summary

In the developing spinal cord, early progenitor cells of the oligodendrocyte lineage are induced in the motor neuron progenitor (pMN) domain of the ventral neuroepithelium by the ventral midline signal *Sonic hedgehog (Shh)*. The ventral generation of oligodendrocytes requires *Nkx6*-regulated expression of the bHLH gene *Olig2* in this domain. In the absence of *Nkx6* genes or *Shh* signaling, the initial expression of *Olig2* in the pMN domain is completely abolished. In this study, we provide the in vivo evidence for a late phase of *Olig* gene expression independent of *Nkx6* and *Shh* gene activities and reveal a brief second wave of oligodendrogenesis in the dorsal spinal cord. In addition, we provide genetic evidence that oligodendrogenesis can occur in the absence of hedgehog receptor *Smoothened*, which is essential for all hedgehog signaling.

Introduction

The spinal cord has served as an excellent model for studying the origin and molecular specification of oligodendrocytes in the developing central nervous system (CNS). Although oligodendrocytes are widely distributed in the adult spinal cord, recent findings have indicated that early progenitors of the oligodendrocyte lineage are induced from specific loci of the ventral neuroepithelium by the ventral midline signal *Sonic hedgehog (Shh)* (for reviews, see Richardson et al., 2000; Spassky et al., 2000; Miller, 2002). Under the influence of *Shh* morphogen, a number of transcription factors are selectively repressed (class I) or induced (class II) in the ventral neural progenitors (Briscoe et al., 2000), with each transcription factor having a different threshold response to the graded *Shh* signaling. As a result, these progenitor transcription factors display a nested pattern of expression along the dorsal-ventral axis. Based on their differential expression in the ventral spinal cord, the ventral neuroepithelium can be divided into five distinct domains (p0–p3 and motor neuron progenitor [pMN]), with each domain expressing a unique combination of progenitor genes and producing a specific neuronal cell type followed by either astrogliogenesis or oligodendrogenesis (Jessell, 2000; Zhou and Anderson, 2002). The pMN domain, which expresses *Nkx6* homeodomain transcription factors (Qiu et al., 1998; Briscoe et al., 2000; Vallstedt et al., 2001) and *Olig* bHLH transcription factors (Mizuguchi et al., 2001; Novitch et al., 2001), first produces motor neurons followed by oligodendrocyte precursor cells (OPCs) (Richardson et al., 1997; Sun et al., 1998; Fu et al., 2002) that subsequently migrate throughout the spinal cord before differentiating into myelinating oligodendrocytes. The sequential generation of motor neurons and OPCs from the pMN domain requires the expression of *Olig1* and *Olig2* transcription factors in this domain, and disruption of the *Olig* genes leads to the loss of both motor neurons and oligodendrocytes in the spinal cord (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Based on these and other observations, it is believed that, in the spinal cord, early OPCs originate from the pMN domain, and oligodendrocyte development is coupled to motor neuron development (Zhou et al., 2001; Lu et al., 2002; Zhou and Anderson, 2002).

The possible contribution of dorsal neuroepithelium to oligodendrocyte development in the spinal cord has been under intensive investigation and considerable debate. In the developing chick embryos, some early transplantation studies suggested that oligodendrocytes were generated from both dorsal and ventral spinal cord (Cameron-Curry and Le Douarin, 1995). However, similar chick-quail grafting experiments argued that dorsal spinal neuroepithelial cells only produced astrocytes but not oligodendrocytes (Pringle et al., 1998). Recent studies in rodents suggested that glial-restricted progenitor (GRP) cells, which can give rise in vitro to OPCs and astrocytes, could be derived from both dorsal and ventral spinal cords (Rao et al., 1998; Gregori et al., 2002). Moreover, in vitro culture of dorsal mouse spinal cord explants, like that of its ventral counterpart, can also produce OPCs, although with a significant delay. In the mean time, the intermediate region located between the dorsal and ventral explants failed to generate OPCs in culture (Sussman et al., 2000), arguing against the possibility of dorsal invasion of OPCs from the ventral region. Together, these experiments indicated that the dorsal spinal neuroepithelial cells in mammals have an intrinsic and independent potential to produce oligodendrocytes under appropriate conditions. However, it is not known whether this potential is realized during the in vivo development of mouse spinal cord, as it has been argued that, in culture, neural progenitor cells may lose their positional cues and behave differently from in vivo in response to exogenous factors (Gabay et al., 2003; Stiles, 2003). For instance, bFGF can ventralize dorsal neural progenitor cells in vitro, resulting in an arbitrary induction *Olig2* expression and oligodendrocyte differentiation (Gabay et al., 2003; Chandran et al., 2003; Kessaris et al., 2004).

To investigate whether OPCs can be derived from the dorsal spinal cord in vivo, we examined oligodendrocyte development in *Nkx6.1*$^{-/-}$ *Nkx6.2*$^{-/-}$ and *Shh*$^{-/-}$ mutant

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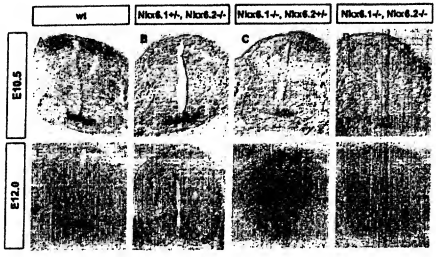

Figure 1. Early Expression of *Olig2* in Various *Nkx6* Mutant Spinal Cords

Transverse spinal cord sections from E10.5 (A–D) and E12.0 (E–H) embryos of various *Nkx* genotypes were subjected to in situ hybridization with *Olig2* riboprobe. The *Olig2* expression in the pMN domain was regulated by the redundant activities of *Nkx6.1* and *Nkx6.2* and was completely suppressed in *Nkx6*$^{-/-}$ double mutants.

spinal cords, in which the early ventral oligodendrogenesis from the pMN domain is abolished, so that the potential dorsal oligodendrogenesis could be unmasked. Our studies on oligodendrogenesis in *Nkx6*$^{-/-}$ double mutants and *Shh*$^{-/-}$ mutants uncovered a transient production of OPCs in the dorsal spinal cord. The dorsal generation of OPCs was also observed in wild-type spinal cords and was confirmed by in vitro culture of dorsal spinal cord explants. Together, these observations suggest an *Nkx*- and *Shh*-independent mechanism for *Olig* gene expression in the dorsal spinal cord after neurogenesis and provide evidence for a late phase of oligodendrogenesis independent of motor neuron development in the dorsal spinal cord.

Results

Nkx6 Dosage-Dependent *Olig* Gene Expression in the Ventral Ventricular Zone during Neurogenesis

Previous studies have demonstrated that *Nkx6.1* and *Nkx6.2* have redundant functions in controlling motor neuron specification, with *Nkx6.1* having a larger effect than *Nkx6.2* (Vallstedt et al., 2001). To examine the effects of different levels of *Nkx6* gene activity on oligodendrocyte development in embryonic spinal cord, we first examined the early expression of *Olig2*, the principal *Olig* gene responsible for motor neuron and oligodendrocyte development (Lu et al., 2002; Takebayashi et al., 2002), in the ventral spinal cords of various *Nkx6* mutants prior to oligodendrogenesis stages. Consistent with the previous findings (Lu et al., 2000; Zhou et al., 2000; Takebayashi et al., 2000), at E10.5 and E12.0, *Olig 2* was exclusively expressed in the pMN domain of the wild-type spinal cord (Figures 1A and 1E). In heterozygous embryos (*Nkx6.1*$^{+/-}$, *Nkx6.2*$^{+/-}$ or *Nkx6.1*$^{+/-}$ *Nkx6.2*$^{+/-}$) and *Nkx6.2*$^{-/-}$ homozygous embryos, *Olig2* expression was not significantly affected (data not shown). In *Nkx6.1*$^{+/-}$ *Nkx6.2*$^{-/-}$ embryos, expression of *Olig2* in the ventral ventricular zone was slightly decreased (Figures 1B and 1F). However, *Olig2* expression was markedly reduced in *Nkx6.1*$^{-/-}$ (Liu et al., 2003) or *Nkx6.1*$^{-/-}$ *Nkx6.2*$^{+/-}$ embryos (Figures 1C and 1G) and completely eliminated in *Nkx6.1*$^{-/-}$ *Nkx6.2*$^{-/-}$ (referred to as *Nkx6*$^{-/-}$ hereafter) compound mutants (Figures 1D and 1H). Collectively, these results indicated a dosage-dependent regulation of *Olig2* expression in the ventral spinal cord by *Nkx6* transcription factors, and its expression is largely dependent on *Nkx6.1* activity but to a lesser extent on *Nkx6.2* activity.

Delayed and Dorsal Expression of *Olig* Genes in *Nkx6*$^{-/-}$ Spinal Cords during Gliogenesis

To investigate whether the lack of *Olig* expression in the pMN domain leads to e complete inhibition of oligodendrogenesis in the spinal cord, we examined OPC generation and differentiation at progressively later stages of embryonic development in *Nkx6*$^{-/-}$ double mutants. At E13.5, many *Olig1+* and *Olig2+* OPCs had already migrated out of the ventral ventricular zone in wild-type spinal cords (Figures 2A and 2C). As expected, no migratory *Olig+* cells were observed outside the ventricular zone in *Nkx6*$^{-/-}$ mutants. Surprisingly, a small number of *Olig1+* and *Olig2+* cells were detected in the mutants in both dorsal and ventral ventricular zone (Figures 2B and 2D). The ventral expression of *Olig* genes in *Nkx6*$^{-/-}$ mutants occurred at approximately the same position as the pMN domain. These data suggested e *Nkx6*-independent regulation of *Olig* gene expression in both the dorsal and ventral spinal cord during oligodendrogenesis stages. The dorsal expression of *Olig* genes in the mutants became more apparent at E14.5, when a small number of *Olig1+* and *Olig2+* OPCs started to

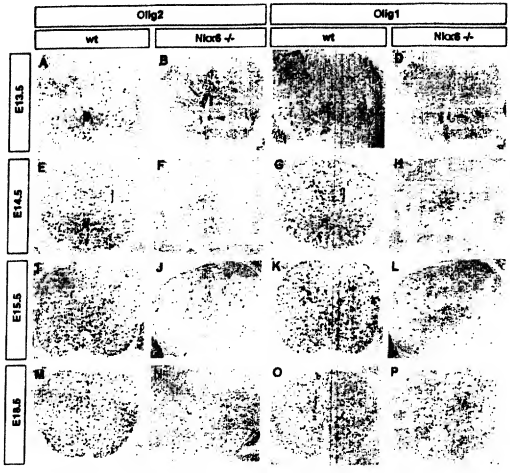

Figure 2. Late and Dorsal Expression of *Olig2* Gene in *Nkx6*$^{-/-}$ Spinal Cords at the Thoracic Level

Transverse sections from E13.5 (A–D), E14.5 (E–H), E15.5 (I–L), and E18.5 (M–P) spinal cords of wild-type and *Nkx6*$^{-/-}$ embryos were subjected to in situ hybridization with riboprobes for *Olig2* and *Olig1*. At E13.5, *Olig1* and *Olig2* expression was upregulated in *Nkx6*$^{-/-}$ double mutants in both dorsal (indicated by arrows) and ventral (indicated by the arrowheads) positions. At E14.5 and later stages, *Olig1*+ and *Olig2*+ cells migrated into the surrounding regions in a dorsal to ventral gradient, in contrast to that seen in the wild-type spinal cords. The positions of dorsal-derived *Olig*+ cells in E14.5 wild-type spinal cord are outlined by a square bracket in (E) and (G).

migrate away from the dorsal ventricular zone (Figures 2F and 2H). Interestingly, very few migratory OPCs were produced from the *Olig*-expressing ventral ventricular zone of the mutant spinal cord. Thus, a vast majority of *Olig*+ OPC cells in *Nkx6*$^{-/-}$ mutants appeared to originate from the dorsal ventricular zone. At this stage, a distinct population of *Olig1*+ and *Olig2*+ cells were also closely associated with the dorsal ventricular or subventricular zone of the wild-type spinal cords (Figures 2E and 2G), and there was an apparent discontinuity between this group of *Olig*+ cells and the ventral-derived *Olig*+ cells (more apparent in Figures 5A, 5I, 8C, and 8E). Together, these observations suggest that a small number of *Olig*+ OPCs are produced from the dorsal neuroepithelial cells in both normal and *Nkx6*$^{-/-}$ spinal cords.

At E15.5, the number of *Olig1/2*+ cells in *Nkx6*$^{-/-}$ mutants was further increased, but most of them remained confined to the dorsal spinal cords (Figures 2J and 2L). In contrast, a higher percentage (55%) of *Olig1/2*+ OPCs were found in the ventral half of wild-type spinal cord (Figures 2I and 2K), and they were presumably derived from the ventral neuroepithelial cells. By E18.5, *Olig1/2*+ cells were distributed more or less evenly throughout the entire spinal cord in *Nkx6*$^{-/-}$ mutants (Figures 2N and 2P), suggesting that the dorsal-derived *Olig*+ OPCs in *Nkx6*$^{-/-}$ mutants migrated progressively from the dorsal to the ventral spinal cord. However, the number of *Olig*+ cells remained significantly smaller than that in wild-type embryos (Figures 2M and 2O; Figure 3Q).

Delayed Appearance of Other Oligodendrocyte Markers in *Nkx6*$^{-/-}$ Mutants

One critical issue for the dorsal-derived *Olig*+ cells is whether they are capable of differentiating further along the oligodendrocyte lineage. To address this question,

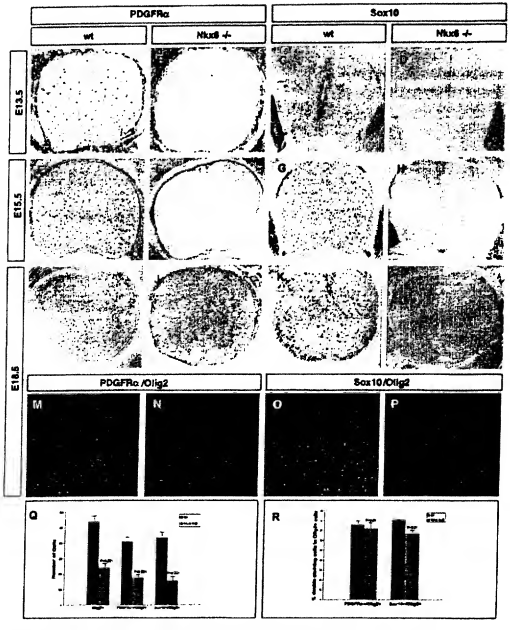

Figure 3. Delayed and Reduced Production of *PDGFRα+* and *Sox10+* OPCs in *Nkx6*$^{-/-}$ Mutant Spinal Cords

(A–L) Spinal cord sections from E13.5 (A–D), E15.5 (E–H), and E18.5 (I–L) wild-type and mutant embryos were subjected to in situ hybridization with *PDGFRα* or *Sox10* riboprobes. A smaller number of *PDGFRα+* and *Sox10+* cells started to emerge in E18.5 *Nkx6*$^{-/-}$ spinal cords.

(M–P) Double immunostaining of E18.5 wild-type and mutant spinal cord with *Olig2* (green) and *PDGFRα* ([M] and [N], red) or *Sox10* ([O] and [P], red).

(Q) The number of Olig2+ single-positive OPCs and *Olig2+/PDGFRα+* or *Olig2+/Sox10+* double-positive cells per spinal cord section in E18.5 wild-type or *Nkx6*$^{-/-}$ mutants (average of three sections).

(R) The percentage of *Olig2+* cells that coexpress *PDGFRα* or *Sox10* in E18.5 wild-type or *Nkx6*$^{-/-}$ mutants. Statistical analyses in (Q) and (R) were performed with Student's t test.

we examined the expression of several oligodendrocytic markers (e.g., *PDGFRα*, *Sox10*, and *MBP*) downstream of *Olig1/2* in *Nkx6*$^{-/-}$ spinal cords. In wild-type spinal cords, expression of *PDGFRα* and *Sox10* is restricted to oligodendrocyte lineage (Pringle et al., 1992; Stolt et al., 2002) and can be detected in the ventral spinal cord at E13.5, whereas their expression in *Nkx6*$^{-/-}$ mutants was not observed until E18.5 (Figures 3A–3L), indicating

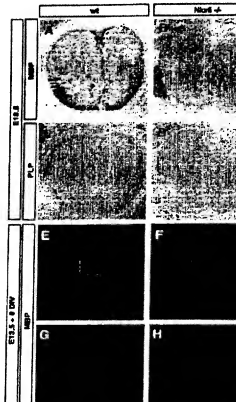

Figure 4. Disrupted Expression of *MBP* and *PLP* in *Nkx6*$^{-/-}$ Mutant Spinal Cords

(A–D) Spinal cord sections were prepared from E18.5 wild-type (A and C) and mutant (B and D) animals and hybridized with *MBP* (A and B) and *PLP* (C and D) riboprobes.

(E–H) Spinal cord explants isolated from E13.5 wild-type and *Nkx6*$^{-/-}$ embryos were cultured on floating membranes for 8 days before they were subjected to anti-*MBP* whole-mount immunostaining. (G) and (H) are higher magnifications of (E) and (F), respectively, showing *MBP*+ myelinating axons.

a significant delay of OPC differentiation. Double immunostaining at this stage confirmed that a high percentage of *Olig2*+ cells in *Nkx6* mutants coexpressed *PDGFRα* and *Sox10* (Figures 3M–3P and 3R), although the total number of *Olig2*+/*PDGFRα*+ and *Olig2*+/*Sox10*+ cells per spinal cord section remained significantly smaller (Figure 3Q). Similarly, expression of the mature oligodendrocyte markers *MBP* and *PLP* in mutant spinal cords was also affected. In normal embryos, many *MBP*+/*PLP*+ oligodendrocytes were seen in the ventral spinal cord at E18.5 (Figures 4A and 4C). However, no *MBP*+/*PLP*+ cells were detected in *Nkx6*$^{-/-}$ mutants at this stage (Figures 4B and 4D). Together, these results suggest that the dorsal derived *Olig*+ cells in *Nkx6*$^{-/-}$ spinal cords can progress along the oligodendrocyte lineage, but they develop and mature much more slowly than the early-born ventral OPCs.

To assess whether dorsal-derived OPCs in *Nkx6*$^{-/-}$ mutants are capable of differentiating into mature oligodendrocytes in vitro, we isolated spinal cord explants from E13.5 wild-type and mutant embryos and cultured them on floating membranes. Following 8 days of culture, a small number of *MBP*+ cells started to emerge in mutant tissues (Figures 4E and 4F). Moreover, *MBP*+ fibers, indicators of myelinating axons, were observed in the axon-enriched medial (ventral) regions of both normal and mutant explants (Figures 4G and 4H). These results suggest that the dorsal-derived OPCs in *Nkx6*$^{-/-}$ mutants are capable of differentiating into *MBP*+ mature oligodendrocytes and form myelin sheaths, at least in vitro.

Olig+ OPCs Are Briefly Produced from the Dorsal Neuroepithelial Cells and Transiently Coexpress Some Dorsal Neural Progenitor Markers

To verify the dorsal origin of a subset of *Olig*+ OPCs in both normal and *Nkx6*$^{-/-}$ spinal cords, sections from E14.5 embryos were subjected to double immunostaining with antibodies against *Olig2* and two dorsal neural progenitor markers, *Pax7* and *Mash1*. During neurogenesis and early gliogenesis, *Pax7* is expressed in the entire dorsal ventricular zone of the spinal cord (Goulding et al., 1993), whereas *Mash1* expression in the dorsal spinal cord is restricted to the dorsal interneuron progenitor domains dI3–dI5 (Gross et al., 2002; Muller et al., 2002; Caspary and Anderson, 2003). Double immunostaining revealed that a subpopulation of *Olig2*+ cells was closely associated with the *Pax7*+ and *Mash1*+ dorsal neuroepithelial cells in both genotypes (Figures 5A–5J). Moreover, a small number of migratory *Olig2*+ cells in the dorsal ventricular zone or immediately adjacent regions coexpressed *Pax7* and *Mash1* (arrows in Figures 5B–5D and 5F–5H; Insets in Figures 5I and 5J). These colabeling data strongly suggested that some *Olig2*+ cells arose from the dI3–dI5 domains of dorsal neural progenitor cells in both normal and *Nkx6*$^{-/-}$ spinal cords and that the dorsal-derived *Olig2*+ cells retained the expression of dorsal markers *Pax7* and *Mash1* for a brief period of time. Although only about 8% of *Olig2*+ cells were also *Pax7*+ in E14.5 wild-type spinal cord, this may be an underestimate of the percentage of dorsal-derived OPC population due to the rapid downregulation of *Pax7* after they migrate away into the surrounding region. Intriguingly, the total number of *Olig2*+/*Pax7*+ and *Olig2*+/*Mash1*+ cells in *Nkx6*$^{-/-}$ double mutants was significantly larger than that in normal embryos (Figure 5M). One plausible explanation is that the dorsal-derived *Olig2*+ cells in mutant spinal cords proliferated more rapidly, possibly due to the lack of competition from the ventral-derived OPCs for mitogens. Alternatively, expression of *Pax7* and *Mash1* in dorsal-derived OPCs may be downregulated more slowly in *Nkx6* mutants.

To confirm our mapping of the origin of dorsal OPCs, we also compared the expression of *Olig2* with that of two other neural progenitor genes, *Dbx1* and *Dbx2*. Previous studies have shown that *Dbx1* is expressed in the dorsal-ventral boundary of the embryonic spinal cord, whereas *Dbx2* is expressed in the dI6 domain of the dorsal spinal cord and the p0 and p1 domains of the ventral spinal cord (Briscoe et al., 2000; Caspary

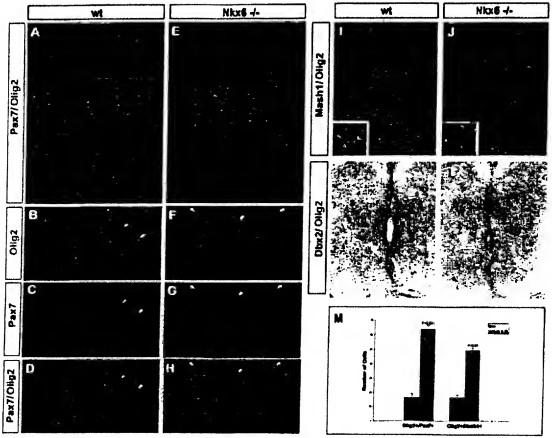

Figure 5. Olig+ Cells Originated from Pax7+ and Mash1+ but Dbx− Dorsal Interneuron Progenitor Domains

(A–H) Coexpression of *Pax7* and *Olig2* in the dorsal spinal cord. E14.5 spinal cord sections from wild-type (A–D) and *Nkx6*$^{-/-}$ (E–H) embryos were simultaneously immunostained with antibodies against *Olig2* (in green) and *Pax7* (in red). (B)–(D) and (F)–(H) are the higher magnifications of the boxed areas in (A) and (E), respectively. In both genotypes, a group of *Olig2*+ cells were produced from the *Pax7*+ dorsal ventricular zone, and some of the *Olig2*+ cells retained the expression of *Pax7* (represented by arrows in [B]–[D] and [F]–[H]).

(I–L) E14.5 wild-type and *Nkx6*$^{-/-}$ spinal cord sections were double immunostained with anti-*Olig2* and anti-*Mash1* antibodies (I and J), or subject to in situ hybridization with *Dbx2* riboprobe followed by anti-*Olig2* immunohistochemistry (K and L). The *Olig2*+/*Mash1*+ double-positive cells are represented in insets in (I) and (J). The dorsal-derived *Olig2*+ cells in (K) and (L) are outlined by a square bracket.

(M) Statistical analyses (Student's *t* test) of *Olig2*+/*Pax7*+ and *Olig2*+/*Mash1*+ double-positive cells in wild-type and *Nkx6*$^{-/-}$ mutants per section.

and Anderson, 2003). Double labeling of E14.5 spinal cord sections demonstrated that the dorsal *Olig2*+ cells lay immediately dorsal to *Dbx2* expression (Figures 5K and 5L) but well above *Dbx1* expression (data not shown), indicating that the dorsal *Olig2*+ cells were derived from regions above the dI6 domain. This result is consistent with the idea that dorsal *Olig2*+ cells are primarily derived from the *Mash1*+ dI3–dI5 dorsal interneuron progenitor cells.

To further confirm that the dorsal spinal cord has an independent potential to generate OPCs, we dissected the dorsal and ventral halves of spinal cord from E11.5 mouse embryos and cultured them separately in collagen gel or on floating membranes in the absence of exogenous bFGF, which is known to induce *Olig2* expression in cultured dorsal neural progenitor cells (Gabay et al., 2003; Chandran et al., 2003). In E11.5 mouse spinal cord, OPCs were not produced from the pMN domain yet (Figures 6A and 6B), excluding the possibility of dorsal invasion of ventral OPCs. Following 3 days of in vitro culture (equivalent to E14.5), a small number of *Olig2*+ cells started to emerge from the dorsal explants and coexpressed Pax7 (Figure 6C). Expression of later OPC markers *PDGFRα* and NG2 in dorsal explants was seen after 4–6 days of culture, and that of mature markers GalC and *MBP* was seen after 6–8 days of culture in vitro (Figures 6E–6L). Together, these data indicated that the dorsal spinal cord explants have an intrinsic potential to produce OPCs, and the schedule of OPC generation and differentiation in dorsal explant culture

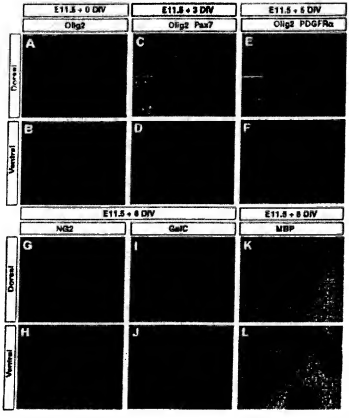

Figure 6. Generation of Oligodendrocytes from Dorsal Spinal Cord Explant Cultures

Mouse E11.5 spinal cord tissues isolated from the thoracic level were bisected into dorsal and ventral halves and cultured separately in collagen gel (A–J) or on floating membrane (K and L) for various days in vitro (DIV) as indicated. Explants were then subject to immunofluorescent staining with antibodies or in situ hybridization with *MBP*.

is similar to that in vivo, in agreement with previous findings (Gabay et al., 2003; Chandran et al., 2003), addition of exogenous bFGF dramatically increased the number of *Olig2*+ OPCs in both dorsal and ventral explants (data not shown).

Generation of Dorsal OPCs in the Absence of *Shh* Signaling

The generation of OPCs in the dorsal spinal cord suggests a *Shh*-independent pathway for oligodendrogenesis, since cell fate specification in the dorsal spinal cord is primarily regulated by dorsal midline signals, notably BMPs (Dickinson et al., 1995; Liem et al., 1995). To test the possibility, we examined whether OPCs' production from dorsal explants can be blocked by anti-*Shh* antibody. In contrast to the previous finding that *Shh* was partially required for O4 expression in dorsal explants (Sussman et al., 2000), we found that anti-*Shh* antibody had no apparent effect on *Olig2* gene expression in dorsal explants, although it dramatically inhibited *Olig2* expression in the ventral explants (Figures 7A–7D). To provide genetic evidence that oligodendrogenesis can occur independent of hedgehog signaling, we differentiated ES (embryonic stem) cells deficient in pan-hedgehog signaling component *Smoothened* (*Smo*$^{-/-}$; Wijgerde et al., 2002) in the presence of retinoic acid and found that GalC+ oligodendrocytes were formed from both normal and *Smo*$^{-/-}$ mutant ES cells at a comparable efficiency (Figures 7E and 7F).

We next examined oligodendrocyte development in *Shh*$^{-/-}$ mutant spinal cord to confirm that *Shh* signaling is not responsible for dorsal oligodendrogenesis in vivo. In *Shh*$^{-/-}$ mutants, the spinal cord is dorsalized, and most of the ventral structures including the pMN domains are missing (Chiang et al., 1996; Pierani et al., 1999). Consistent with some earlier findings that *Shh* is required for ventral oligodendrogenesis (Lu et al., 2000; Alberta et al., 2001), no early *Olig1/2*+ OPCs were produced in *Shh* mutant spinal cords at or before E13.5 (Figures 8A and 8B; data not shown). However, at E14.5, a small number of *Olig1*+ and *Olig2*+ cells started to appear in the dorsal region of the mutant spinal cords (Figures 8D and 8F). By E18.5, a larger number of *Olig2*+ cells were observed throughout the mutant spinal cord (Figure 8H).

Similar to our data in *Nkx6*$^{-/-}$ mutants, oligodendrocyte lineage progression in *Shh* null spinal cord was also delayed. Expression of *PDGFR* and *Sox10* was not detected until E18.5 (Figures 8I–8L), and no *MBP* expression was observed at perinatal stages (Figures 8M and 8N). However, when spinal cord explants isolated from E18.5 mutant embryos were cultured in vitro for 2 additional days, a small number of *MBP*+ cells started to emerge in mutant tissues (Figures 8O and 8P), indicating

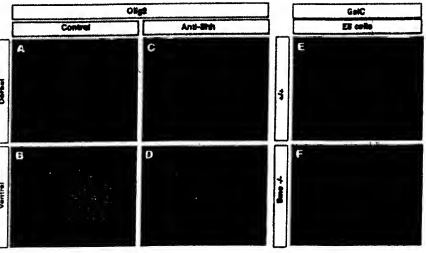

Figure 7. *Shh*-independent Generation of Oligodendrocytes in Spinal Explants and ES Cells

(A–D) Inhibition of *Olig* gene expression by anti-Shh antibody in ventral explants but not in dorsal explants. Dorsal and ventral spinal cord explants from E11.5 wild-type embryos were cultured in collagen gel for 3 days in the absence (A and B) or presence (C and D) of anti-*Shh* antibody prior to immunostaining with anti-*Olig2*.

(E and F) Differentiation of wild-type (E) and *Smo*$^{-/-}$ (F) ES cells into GalC+ oligodendrocytes.

that the dorsal-derived OPCs in *Shh* mutants are able to differentiate into *MBP*+ mature oligodendrocytes as well.

Discussion

This study reveals an *Nkx6*- and *Shh*-independent mechanism for a late phase of *Olig* gene expression in the dorsal spinal cord after the onset of early oligodendrogenesis from the pMN domain (Figure 9). The late *Olig* gene expression is associated with a brief wave of oligodendrogenesis in the dorsal spinal cord. These findings provide evidence for multiple origins of OPC generation involving distinct inductive signals during spinal cord development.

Nkx6-Independent Mechanisms for *Olig* Gene Expression and Oligodendrogenesis in the Spinal Cord

Previous work had demonstrated that *Nkx6.1* and *Nkx6.2* have redundant functions in controlling motor neuron specification in the spinal cord (Vallstedt et al., 2001). Consistent with this line of study, our findings indicate that *Nkx6.1* and *Nkx6.2* have redundant activities in regulating the early expression of *Olig2* in the pMN domain, with *Nkx6.1* exerting a larger effect than *Nkx6.2* (Figure 1). In the absence of both *Nkx6.1* and *Nkx6.2*, the initial expression of *Olig 2* in the pMN domain is completely abolished. In keeping with the idea that early progenitors of the oligodendrocyte lineage are derived from the *Olig2*+ pMN domain of the ventral spinal cord, the loss of *Olig2* expression in the pMN domain in *Nkx6*$^{-/-}$ mutants was associated with the failure of production of early OPC cells from the ventral spinal cord (Figure 2).

Despite the lack of early expression of *Olig2* in the pMN domain in *Nkx6*$^{-/-}$ mutants, a low level of *Olig1* and *Olig2* expression started to be detected in both the ventral and dorsal ventricular zone after the onset of oligodendrogenesis (Figure 2). In the dorsal spinal cord, *Olig* gene expression was detected in *Pax7*+/*Mash1*+ dI3–dI6 dorsal neural progenitor domains starting at E13.5 (Figures 2 and 5). In the ventral spinal cord, a small number of ventral ventricular cells started to express *Olig1* and *Olig2* genes at approximately the same position as the pMN domain (Figures 2B and 2D). Together, these results indicate an *Nkx6*-independent regulation of *Olig* gene expression in both the dorsal and ventral spinal cord. The late phase of *Olig* gene expression in the dorsal spinal neuroepithelium was also observed in wild-type spinal cord, mostly at E14.5 (Figures 5 and 8) but occasionally at E13.5 (data not shown). Thus, the dorsal ventricular *Olig2* expression appeared to be slightly advanced or enhanced in *Nkx6* mutants. This enhancement might partially account for the increased population of *Olig2*+/*Pax7*+ cells in the mutants. Interestingly, no *Olig* expression was observed in E13.5 *Shh* mutants (Figure 8), suggesting that the premature or enhanced dorsal ventricular *Olig* expression was not simply due to the loss of ventral patterning, but more specifically associated with the absence of *Nkx6* gene expression.

The dorsal *Olig* gene expression was associated with a transient production of OPCs starting at around E14.5, about 2 days later than the ventral oligodendrogenesis from the pMN domain (Figure 9B). In both wild-type and *Nkx6*$^{-/-}$ spinal cords, migratory *Olig1/2*+ OPCs were briefly produced from the dorsal neural progenitor cells. Several lines of evidence strongly suggest that these OPC cells are generated de novo from the dorsal ventricular cells, instead of having migrated up from the ventral cord. First, many dorsal *Olig2*+ cells coexpressed sev-

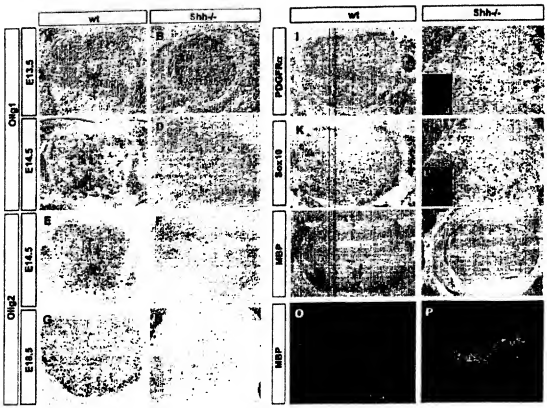

Figure 8. Oligodendrocyte Development in *Shh* Mutant Spinal Cord

(A–H) Dorsal generation of *Olig1/2+* OPC cells in *Shh* mutant spinal cords. Spinal cord sections from E13.5 (*A* and *B*), E14.5 (*C–F*), and E18.5 (*G* and *H*) wild-type (*A*, *C*, *E*, and *G*) or *Shh*$^{-/-}$ (*B*, *D*, *F*, and *H*) embryos were subjected to in situ hybridization (ISH) with *Olig1* (*A–D*) and *Olig2* (*E–H*) riboprobes. At E13.5, *Olig+* OPCs were generated from the ventral neuroepithelium in wild-type embryos but not in *Shh* mutants. At E14.5, a small group of *Olig1+* and *Olig2+* cells were associated with the dorsal neuroepithelium in both wild-type and *Shh* mutants. Dorsal *Olig+* cells are outlined by a square bracket in (*C*) and (*E*).

(I–N) Distribution and differentiation of OPC cells in *Shh* mutant spinal cords. (I–N) Expression of *PDGFRα* (*I* and *J*), *Sox10* (*K* and *L*), and *MBP* (*M* and *N*) in E18.5 wild-type (*I*, *K*, and *M*) and *Shh* mutant (*J*, *L*, and *N*) spinal cords. *Olig2+/PDGFRα+* and *Olig2+/Sox10−* cells in mutants are represented in insets (*J′*) and (*L′*), respectively.

(O and P) Spinal cord tissues from E18.5 wild-type and *Shh* mutant embryos were isolated and cultured on polycarbonate membranes for 2 days in vitro and then subjected to whole-mount ISH with the *MBP* probe. A small number of *MBP+* cells emerged in the mutant tissue.

eral dorsal neural progenitor genes such as *Pax7* and *Mash1* (Figure 5). Second, dorsal neural explants isolated from E11.5 spinal cord prior to ventral oligodendrogenesis can give rise to OPCs and oligodendrocytes on schedule as in vivo (Figure 6). Third, dorsal *Olig2* expression in *Mcrl* mutants was no later than its ventral expression. At E13.5 and E14.5, there was an apparent discontinuity of dorsal *Olig2+* cells and ventral *Olig2+* cells in the mutants (Figures 2B, 2D, 5E, and 5J), and the number of *Olig2+* cells in E14.5 dorsal half far exceeded that of ventral *Olig2+* cells, arguing against the dorsal migration of *Olig2+* cells at least at these early stages. However, it is plausible that some ventral *Olig2+* cells could migrate dorsally after E14.5 and contribute to the dorsal OPC population. Finally, OPCs can be produced from the dorsal region of *Shh* mutant spinal cord, which lacks the pMN domain (Pierani et al., 1999) and presumably the ventral oligodendrogenesis (Figure 8), although we can not absolutely exclude the possibility that a few *Olig+* cells could also be generated from the remaining p0 and p1 domains in the most ventral region (Pierani et al., 1999). Together, these observations indicate that dorsal *Olig2+* cells can be produced locally from the dorsal neuroepithelial cells in normal and *Mcrl/Shh* mutant spinal cords. Since the dorsal *Olig2+/Pax7+/Mash+* OPCs were observed in the thoracic or even more caudal regions (data not shown), it is unlikely that they represent the longitudinally migrating cells from the rostral hindbrain.

Similar to the early ventral OPCs, the dorsal-derived *Olig+* OPCs are capable of migration, proliferation, and differentiation along the oligodendrocytic lineage after they migrate out of the germinal zone. The delayed appearance of *PDGFRa+* and *Sox10+* OPCs in the *Mcrl*$^{-/-}$ and *Shh*$^{-/-}$ mutants indicated that the late-born dorsal OPCs develop and differentiate much later than the early-born ventral oligodendrocytes. Although no *MBP* and *PLP* expression was observed in both mutants at

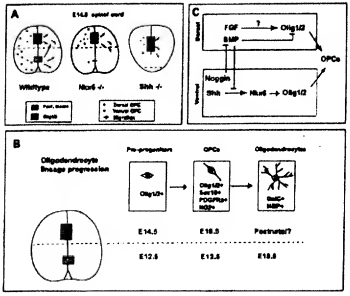

Figure 6. Proposed Origins and Molecular Specification of Oligodendrocytes in the Spinal Cord

(A) Proposed oligodendrocyte development in *Nkx6*$^{-/-}$ and *Shh*$^{-/-}$ mutant spinal cords. In the wild-type, a vast majority of OPCs are derived from the ventral pMN domain. A subpopulation of OPCs is also generated from the dorsal d83–d85 domains independent of *Nkx6* and *Shh* activities. The arrows represent the possible migration directions.

(B) Time schedule of lineage progression for both dorsal- and ventral-derived OPCs. The generation of dorsal OPCs is about 2 days later than that of ventral OPCs. In general, there is a parallel delay in dorsal OPC generation and their differentiation.

(C) Proposed molecular pathways for dorsal and ventral oligodendrogenesis. *Shh* and *BMPs* are known to be the major inducer and repressor of oligodendrogenesis, respectively. In the ventral spinal cord, OPCs are generated in a *Shh*/*Nkx6*-dependent mechanism. Repression of *BMP* signaling by the notochord-derived Noggin may also contribute to ventral oligodendrogenesis. In the dorsal spinal cord, OPCs are generated independent of the *Shh*/*Nkx6* pathway and may result from a combination of FGFs and progressive loss of BMP inhibition over time.

E18.5, OPCs in both mutants could mature into *MBP*+ oligodendrocytes (Figures 4F and 8F) or even myelinate axons (Figure 4H) if they were allowed to develop further in vitro. Consistently, OPCs generated in the dorsal explants of normal embryos could also differentiate into mature oligodendrocytes (Figure 6). In general, there appears to be a parallel delay of OPC generation and their terminal differentiation as observed in other genetic mutants (Qi et al., 2003; Liu et al., 2003).

A *Shh*-Independent Pathway for Oligodendrogenesis in the Developing Spinal Cord

Early studies demonstrated that blockade of *Shh* signaling can inhibit oligodendrogenesis both in vivo and in vitro (Orentas et al., 1999; Davies and Miller, 2001; Tekki-Kessaris et al., 2001). Thus, it has been believed that *Shh* signaling is required for the development of oligodendrocytes in the entire CNS. However, the observations that OPCs can be produced from dorsal spinal cord explants in the presence of anti-*Shh* antibody (Figure 7) or from dissociated dorsal neural progenitor cells in the presence of bFGF and cyclopamine (Chandran et al., 2003; Kessaris et al., 2004) have suggested a *Shh*-independent pathway for oligodendrogenesis. However, the efficiency and specificity of the antibody and cyclopamine inhibition could potentially lead to alternative explanations. Our observation that GalC+ oligodendrocytes can develop from *Smo*$^{-/-}$ mutant ES cells provides unambiguous genetic evidence that oligodendrogenesis can occur in the absence of hedgehog signaling (Figure 7), at least in vitro.

Despite the in vitro data for *Shh*-independent oligodendrogenesis, there has been no evidence that this phenomenon can be applied to in vivo development. Our findings that OPCs are generated from *Shh*$^{-/-}$ spinal cord provide the missing link that *Shh*-independent oligodendrogenesis also occurs during spinal cord development as well. In the absence of *Shh* signaling, *Olig1/2*+ OPCs were still generated on schedule (at E14.5) as in the wild-type dorsal spinal cord. Although we can not formally exclude the possibility that the hedgehog signaling in *Shh*$^{-/-}$ mutants could be compensated by the upregulation of expression of other hedgehog members such as *Indian hedgehog* (*Ihh*) or *Desert hedgehog* (*Dhh*), we do not favor this possibility, as we failed to detect by in situ hybridization (ISH) the expression of *Ihh* or *Dhh* in either wild-type or *Shh* mutant spinal cords around the onset of dorsal oligodendrogenesis (data not shown).

The signaling mechanism underlying the *Shh*-independent late phase of dorsal oligodendrogenesis in the spinal cord is uncertain at this stage. Since bFGF can induce oligodendrocytes in dissociated dorsal neural progenitor cells (Gabay et al., 2003) independent of *Shh* signaling (Chandran et al., 2003; Kessaris et al., 2004) and in dorsal explants (our unpublished data), it is conceivable that *FGF* signaling may be partially responsible for the late production of OPCs in the dorsal spinal cord (Figure 9C). In addition, the progressive reduction of *BMP* signaling over time may also contribute to dorsal oligodendrogenesis. It is known that BMP can antagonize *Shh*-induced oligodendrocyte specification, and experimental inhibition of *BMP* signaling is sufficient to induce oligodendrocyte production both in vivo and in vitro (Mekki-Dauriac et al., 2002; Miller et al., 2004; Vallstedt et al., 2005 [this issue of *Neuron*]). Future studies on the expression and function of various FGF and BMP molecules and their receptors will be needed to determine their possible in vivo roles in the late phase of oligodendrogenesis in the dorsal neural progenitor cells.

Multiple Origins and Phases of Oligodendrogenesis in the Developing Spinal Cord

It is generally accepted that early OPCs are induced from the pMN domain of ventral spinal cord by the *Shh* signal (Poncet et al., 1996; Pringle et al., 1996; Orentas et al., 1999) and that oligodendrocyte development is coupled to motor neuron development (Richardson et al., 2000; Lu et al., 2002; Zhou and Anderson, 2002). However, our data in *Nkx6*$^{-/-}$ and *Shh*$^{-/-}$ mutants and in wild-type embryos as well have provided strong evidence that a subset of OPCs originate from the dorsal spinal cord independent of motor neuron development at later stages of oligodendrogenesis. Therefore, there are multiple origins of, and distinct inductive mechanisms for, OPC production in the developing mammalian spinal cord. Based on these observations, we propose that there are two phases of *Olig* gene expression during normal spinal cord development, the *Shh*/*Nkx6*-dependent early phase of *Olig* expression and oligodendrogenesis in the pMN domain and the *Shh*/*Nkx6*-independent late phase of *Olig* expression and oligodendrogenesis in the dorsal spinal cord (Figures 9A and 9B).

The dorsal oligodendrogenesis in mouse spinal cord has long been unnoticed, because the production of OPC cells from the dorsal spinal cord is both late and transient (at around E14.5) as compared to the early OPC production (E12.5) from the ventral spinal cord. By the time OPCs are being generated from the dorsal spinal cord, a large number of ventral-derived OPCs have already invaded into the dorsal spinal cord and thus mask the existence of the late-born dorsal OPCs. Only in mutants (e.g., *Nkx6*$^{-/-}$ and *Shh*$^{-/-}$) in which the ventral oligodendrogenesis from the pMN domain is inhibited or greatly compromised can the dorsal generation of OPCs be uncovered.

Experimental Procedures

Genotyping of *Nkx6* Mutant Mice

The *Nkx6.1* and *Nkx6.2* homozygous null embryos were obtained by the interbreeding of double heterozygous animals. Genomic DNA extracted from embryonic tissues or mouse tails was used for genotyping by Southern analysis or by PCR. Genotyping of *Nkx6.1* and *Nkx6.2* loci was described in Sander et al. (2000) and Cai et al. (2001), respectively. Genotyping of *Shh* mutant mice was carried out according to Chiang et al. (1996).

In Situ RNA Hybridization and Immunohistochemical Staining

Spinal cord tissues at the thoracic level were isolated from E10.5 to E18.5 mouse embryos and then fixed in 4% paraformaldehyde at 4°C overnight. Following fixation, tissues were transferred to 20% sucrose in PBS overnight, embedded in OCT media, and then sectioned (20 μm thickness) on a cryostat. Adjacent sections from the wild-type and mutant embryos were subsequently subjected to ISH or immunofluorescent staining. ISH was performed as described in Schaeren-Wiemers and Gerfin-Moser (1993) with minor modifications, and the detailed protocol is available upon request. Double immunofluorescent procedures were previously described in Xu et al. (2000). For the combination of ISH and immunohistochemistry, sections were first subject to ISH with *Dbx2* riboprobe, rinsed several times with PBS followed by immunohistochemical staining with anti-*Olig2*, Anti-*Pax7* (1:50 DSHB), anti-*Msx1* (1:200), anti-NG2 (1:1500), anti-*PDGFRα* (1:300), anti-*MBP* (1:5000), and anti-GalC (1:50) were obtained from commercial sources. Anti-*Olig2* (1:3000) and anti-*Sox10* (1:3000) polyclonal antibodies were generously provided by Drs. Chuck Stiles and Michael Wegner.

Spinal Cord Explant Culture

Segments of spinal cord tissues were isolated from E11.5, E13.5, or E18.5 embryos at the thoracic region and grown either in collagen gel or on 8.0 μm nucleopore polycarbonate membranes (Costar) floating on culture medium (DMEM + N2 supplement + 30 ng/ml T3 + 40 ng/ml T4 + 1 mg/ml BSA + 0.5% FBS + Pen-Strept. In our experience, the inclusion of a small amount of FBS in culture medium made cells healthier and did not appear to significantly affect oligodendrocyte development in explant culture as compared to no serum (data not shown), although serum was shown to inhibit OPC differentiation (Raff et al., 1983). For anti-*Shh* antibody treatment, 5E1 supernatant (1:3 DSHB) was added to culture medium. Following various days of culture in vitro, explants were then fixed in 4% PFA and processed for immunofluorescent staining (Xu et al., 2000) or whole-mount in situ RNA hybridization with *MBP* riboprobe, as described in Cai et al. (1999).

Culture and Differentiation of Embryonic Stem Cells

Normal and *Smo*$^{-/-}$ ES cells were maintained on MEF feeder cells in ES medium with LIF. During differentiation, ES cells were dissociated and grown on nonadherent petri dishes for 2 days in the absence of LIF and 4 additional days in 5 μM retinoic acid to generate embryoid bodies (EB). Following 10 days of suspension culture, EBs were trypsinized with trypsin/EDTA, and cells were plated on laminin-coated cover slips and cultured for 15 days prior to immunofluorescent staining with anti-GalC antibody.

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EVIDENCE APPENDIX
EXHIBIT 12

Pringle et al., "*Fgfr3* Expression by Astrocytes and Their Precursors: Evidence That Astrocytes and Oligodendrocytes Originate in Distinct Neuroepithelial Domains," *Development* 130:93-102 (2003)

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Fgfr3 expression by astrocytes and their precursors: evidence that astrocytes and oligodendrocytes originate in distinct neuroepithelial domains

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SUMMARY

The postnatal central nervous system (CNS) contains many scattered cells that express fibroblast growth factor receptor 3 transcripts (*Fgfr3*). They first appear in the ventricular zone (VZ) of the embryonic spinal cord in mid-gestation and then distribute into both grey and white matter – suggesting that they are glial cells, not neurones. The *Fgfr3*$^+$ cells are interspersed with but distinct from platelet-derived growth factor receptor α (*Pdgfra*)-positive oligodendrocyte progenitors. This fits with the observation that *Fgfr3* expression is preferentially excluded from the pMN domain of the ventral VZ where *Pdgfra*$^+$ oligodendrocyte progenitors – and motoneurones – originate. Many glial fibrillary acidic protein (Gfap)-positive astrocytes co-express *Fgfr3* in vitro and in vivo. *Fgfr3*$^+$ cells within and outside the VZ also express the astroglial marker glutamine synthetase (*Glns*). We conclude that (1) *Fgfr3* marks astrocytes and their neuroepithelial precursors in the developing CNS and (2) astrocytes and oligodendrocytes originate in complementary domains of the VZ. Production of astrocytes from cultured neuroepithelial cells is hedgehog independent, whereas oligodendrocyte development requires hedgehog signalling, adding further support to the idea that astrocytes and oligodendrocytes can develop independently. In addition, we found that mice with a targeted deletion in the *Fgfr3* locus strongly upregulate Gfap in grey matter (protoplasmic) astrocytes, implying that signalling through Fgfr3 normally represses Gfap expression in vivo.

Key words: Fgfr3, Targeted deletion, Astrocyte, Reactive gliosis, CNS, Neuroepithelium

INTRODUCTION

In the embryonic CNS, neurones and glia develop from the neuroepithelial cells of the ventricular zone (VZ) that surrounds the ventricles of the brain and the lumen of the spinal cord. Different domains of the VZ express different gene products and generate different subsets of neurones and/or glia. For example, the ventral half of the spinal cord VZ is subdivided into five regions labelled (from ventral to dorsal) p3, pMN, p2, p1 and p0. These five domains express different combinations of homeodomain (HD) and basic helix-loop-helix (bHLH) transcription factors and generate distinct classes of spinal neurones; pMN gives rise to somatic motoneurones, whereas p0-p3 give rise to four classes of ventral interneurones (V0-V3 respectively) (reviewed by Briscoe and Ericson, 1999; Jessell, 2001). In the brainstem, p3 also gives rise to visceral motoneurones (Ericson et al., 1997).

After neurones, the VZ switches to producing glial cells. Oligodendrocytes, the myelinating glial cells of the CNS, develop from the ventral VZ. Small numbers of oligodendrocyte progenitors (OLPs), which express the platelet-derived growth factor receptor-α (Pdgfra), first appear at the ventricular surface on embryonic day 12.5 (E12.5) in the mouse, then proliferate and migrate away into the grey and white matter before starting to differentiate into myelin-forming oligodendrocytes (Miller, 1996; Rogister et al., 1999; Richardson et al., 2000; Spassky et al., 2000). In rodents, OLPs are generated from the same part of the neuroepithelium as somatic motoneurones (MNs) but not until after MN production has ceased (Sun et al., 1998; Lu et al., 2000) (for a review, see Rowitch et al., 2002). This prompted us to suggest that there is a pool of shared neuroglial precursors that first generates MNs, then switches to OLPs (Richardson et al., 1997; Richardson et al., 2000). This idea has been supported recently by the finding that the bHLH proteins Olig1 and Olig2 are expressed and required in pMN for production of both motoneurones and OLPs (Lu et al., 2002; Zhou and Anderson, 2002; Takebayashi et al., 2002) (reviewed by Rowitch et al., 2002).

Where do astrocytes, the other major class of CNS glia, originate in the neuroepithelium? It is believed that at least some astrocytes are generated by transdifferentiation of radial

glia (Bignami and Dahl, 1974; Choi et al., 1983; Benjelloun-Touimi et al., 1985; Voigt, 1989; Culican et al., 1990). Others are formed from multipotent precursors in the subventricular zones (SVZ) of the postnatal brain. However, the origins of astrocytes in the developing spinal cord are unclear, so we looked for an astrocyte lineage marker that might be helpful in following the development of astrocytes from their earliest precursors in the VZ. Previous expression studies of the fibroblast growth factor receptor 3 (*Fgfr3*) suggested that this receptor might be expressed in glial cells, possibly astrocytes (Peters et al., 1993; Miyake et al., 1996). Our own studies, reported here, support this conclusion and suggest that *Fgfr3*-positive astrocytes develop from *Fgfr3*-positive precursor cells in the neuroepithelium. *Fgfr3* is not expressed equally in all parts of the neuroepithelium but is reduced or absent from pMN, suggesting that astrocytes and OLPs have separate neuroepithelial origins. We also found that astrocytes are formed in vitro in the absence of hedgehog signalling – unlike oligodendrocytes, which require sonic hedgehog from the ventral midline. This reinforces the notion that at least some astrocytes develop independently of OLPs.

To investigate the function of *Fgfr3* in astrocytes, we examined mice with a targeted deletion in the *Fgfr3* locus (Colvin et al., 1996). The number of *Fgfr3*-expressing cells was normal in the knockout, suggesting that *Fgfr3* does not mediate a mitogenic or survival-promoting effect for these cells. However, Gfap was markedly upregulated in grey matter astrocytes, which normally have little or no Gfap – unlike their counterparts in white matter. Our results imply that signalling through Fgfr3 normally represses Gfap expression in grey matter astrocytes and suggest that white matter astrocytes might preferentially express Gfap because ligands for Fgfr3 are not normally available in axon tracts.

MATERIALS AND METHODS

Tissue and cell cultures

Spinal cords from stage 12-13 (48 hour) chick embryos were dissected into dorsal, middle and ventral thirds using a flame-sharpened tungsten needle. Tissue fragments were cultured as explants in collagen gels (Guthrie and Lumsden, 1994) in defined BS medium (Bottenstein and Sato, 1979) containing 0.25% (v/v) foetal bovine serum (FBS) and conalbumin in place of transferrin (Pringle et al., 1996).

For dissociated cell cultures, E17 rat cervical spinal cords were digested in 0.25% (w/v) trypsin in Earle's buffered saline (Ca^{2+} and Mg^{2+} free; Gibco) for 15 minutes at 37°C, then FBS was added to a final concentration of 10% (v/v) and the tissue physically dissociated by trituration. Cells were washed by centrifugation and resuspended in BS medium before plating in a 50 µl droplet on poly-D-lysine-coated glass coverslips (5×10^4 cells/coverslip). Both explants and dissociated cell cultures were cultured at 37°C in 5% CO_2 in a humidified atmosphere.

Neutralising Shh activity in vitro

Monoclonal Shh neutralising antibody 5E1 (Ericson et al., 1996) was concentrated by ammonium sulphate precipitation from hybridoma supernatants (Harlow and Lane, 1988). Monoclonal anti-NG2 proteoglycan #4.11 (Stallcup and Beasley, 1987) was used as a negative control. Precipitated antibodies were dissolved in a small volume of PBS and dialysed first against PBS and then Dulbecco's modified Eagle's medium (DMEM, Gibco). The final volumes were approximately tenfold less than the starting volumes and were assumed to be ten time as concentrated. Explants were incubated in the presence of either anti-Shh or control antibodies at twice the final concentration. Antibodies were added at the start of the experiment and fresh medium and antibody were added each day thereafter. In some experiments cyclopamine (1 µM; from William Gaffield) instead of anti-Shh was added to cultures daily.

BrdU labelling in vivo

E18 pregnant mice were injected intraperitoneally with BrdU at 50 µg BrdU per gram bodyweight. Two injections were given, 6 hours apart. Mice were sacrificed 3 hours after the second injection and the embryos were processed for *Fgfr3* in situ hybridisation and BrdU immunolabelling.

Preparation of tissue sections

C57Bl/6 mice were obtained from Olac and bred in-house. Noon on the day of discovery of the vaginal plug was designated embryonic day 0.5 (E0.5). We also used *Fgfr3*-null mice (Colvin et al., 1996) bred at UCL. Mid-gestation embryos were staged according to the morphological criteria of Theiler (Theiler, 1972). Rats (Sprague-Dawley) were obtained from the UCL breeding colony and staged according to Long and Burlingame (Long and Burlingame, 1938). Fertilised White Leghorn chicken eggs were obtained from Needle Farm (Cambridge, UK). They were incubated at 38°C and the chicken embryos staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Embryos were decapitated and immersion-fixed in cold 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 24 hours before cryoprotecting in cold 20% (w/v) sucrose in PBS for at least 24 hours. In sections processed for immunohistochemistry after in situ hybridisation, the fixation time was reduced to 1 hour to preserve epitope integrity. Tissues were immersed in OCT embedding compound (BDH), frozen on solid CO_2 and stored at −70°C before sectioning. Frozen sections (15 µm) were cut on a cryostat and collected on 3-aminopropyl-triethoxysilane (APES)-coated glass microscope slides. Sections were air-dried for 2 hours before storing dry at −70°C.

Immunohistochemistry

Anti-Gfap monoclonal ascites, clone G-A-5 (Sigma), was used at a dilution of 1:400. Anti-BrdU (monoclonal BU20?) (Magaud et al., 1989) was used at 1:5 dilution. Monoclonal O4 (Sommer and Schachner, 1981) was used as cell culture supernatant diluted 1:5. Secondary antibodies were rhodamine- or fluorescein-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin (all from Pierce) diluted 1:200. All antibodies were diluted in PBS containing 0.1% (v/v) Triton X-100 and 10% (v/v) normal goat serum, except O4, which was diluted in PBS alone. Sometimes diaminobenzidine (DAB) labelling (ABC kit, Vector Laboratories) was used instead of fluorescence detection.

In situ hybridisation

Our in situ hybridisation procedures have been described (Pringle et al., 1996; Fruttiger et al., 1999); detailed protocols are available at http://www.ucl.ac.uk/~ucbzwdr/richardson.htm. Digoxigenin (DIG)- or fluorescein (FITC)-labelled RNA probes were transcribed in vitro from cloned cDNAs. The rat *Fgfr3* probe was transcribed from a ~900 bp partial cDNA encoding most of the tyrosine kinase (TK) domain (W.-P. Yu, PhD thesis, University of London, 1995) and the chicken *Fgfr3* probe from a ~440 bp partial cDNA encoding part of the TK domain (from Ivor Mason, King's College London). The mouse *Pdgfra* probe was made from a ~1600 bp cDNA encoding most of the extracellular domain (from Chiayeng Wang, University of Chicago). The chicken *Pdgfra* probe was made from a ~3200 bp cDNA covering most of the 3' untranslated region of the mRNA (from Marc Mercola, Harvard Medical School, Boston).

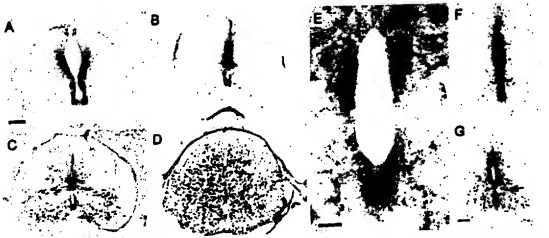

Fig. 1. *Fgfr3* expression in transverse sections of embryonic chick and mouse cervical spinal cords. (A) Chick stage 22-24 (E3.5-4); (B) chick stage 34 (E8); (C) chick stage 35 (E9); (D) chick stage 37 (E11); (E) chick stage 35 (E9); (F) mouse E13.5; and (G) mouse E14.5. Initially, *Fgfr3* is expressed in the floor plate and the ventral two-thirds of the VZ (A) and is later downregulated in part of the ventral VZ (B). Starting around stage 34 (E8) *Fgfr3*+ cells are visible in the parenchyme of the cord. By stage 37 (E11) the floor plate and VZ no longer express *Fgfr3* but scattered *Fgfr3*+ cells are present throughout most of the cross-section of the cord, including both grey and white matter (D). (E) A magnified image of the ventral VZ from a stage 35 (E9) cord, showing the two spatially separated domains of *Fgfr3* expression. A similar progression occurs in mouse (F,G). However, the ventral 'gap' is not so pronounced in mouse (arrow in G). Scale bars: 200 μm (A-D), 100 μm (F,G), 50 μm (E).

For double in situ hybridisation, two probes – one FITC labelled and the other DIG labelled – were applied to sections simultaneously. The FITC signal was visualised with alkaline phosphatase (AP)-conjugated anti-FITC Fab_2 fragments before developing in p-iodonitrotetrazolium violet (INT) and 5-bromo-4-chloro-3-indolyl phosphate (toluidine salt) (BCIP), which produces a magenta/brown reaction product. The sections were photographed, then the AP was inactivated by heating at 65°C for 30 minutes followed by incubating in 0.2 M glycine (pH 2) for 30 minutes at room temperature. The INT-BCIP reaction product was removed by dehydration through graded alcohols, concluding with 100% ethanol for 10 minutes at room temperature. The DIG signal was then visualised with AP-conjugated anti-DIG Fab_2 fragments and a mixture of nitroblue tetrazolium (NBT) and BCIP (all reagents from Roche Molecular Biochemicals) and the sections re-photographed. No labelling with NBT/BCIP was observed when we omitted either the DIG labelled probe or the anti-DIG antibody (data not shown).

For the *Fgfr3-Pdgfra* double in situ hybridisation of Fig. 4 we visualised the FITC (*Pdgfra*) signal with horseradish peroxidase (HRP)-conjugated anti-FITC Fab_2 fragments (Roche) before developing in fluorescein-tyramide reagent (NEN™ Life Science Products, Boston) according to the manufacturer's instructions. The HRP-conjugate was inactivated by incubating in 2% (v/v) hydrogen peroxide for 30 minutes at room temperature. The DIG (*Fgfr3*) signal was then visualised with HRP-conjugated anti-DIG Fab_2 fragments followed by rhodamine-tyramide, and the sections photographed under fluorescence optics. As specificity controls we omitted either the FITC-labelled *Pdgfra* probe or the HRP-conjugated anti-FITC antibody, which gave no staining other than for *Fgfr3* (not shown).

Combined immunolabelling and in situ hybridisation

For the experiment of Fig. 7, cultured cells were first subjected to in situ hybridisation with a [^{35}S]-labelled RNA probe against *Fgfr3* then immunolabeled with anti-Gfap and biotinylated goat-anti-mouse Ig. The Gfap signal was developed with DAB and the slides dehydrated through ascending alcohols, dipped in nuclear emulsion (Ilford K5), exposed in the dark for several days and developed in Kodak D19.

RESULTS

Fgfr3 expression in the embryonic spinal cord

We examined *Fgfr3* expression in the embryonic chick spinal cord by in situ hybridisation. At stage 22-24 (corresponding to ~E4), *Fgfr3* expression was confined to the floor plate and the ventral two-thirds of the VZ (Fig. 1A). By stage 34 (E8) *Fgfr3* expression had been extinguished in part of the ventral VZ so that a gap developed in the expression pattern (e.g. Fig. 1B).

Individual *Fgfr3*+ cells were also present outside the VZ

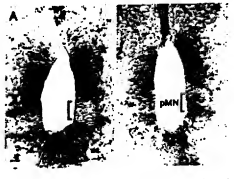

Fig. 2. Expression of *Fgfr3* and *Olig2*. Transverse sections through stage 35 (E9) chicken spinal cords were subjected to in situ hybridisation for *Fgfr3* (A) or double in situ for *Fgfr3* and *Olig2* (B). At this age, *Fgfr3* expression is confined to the VZ and a few scattered cells outside the VZ. The two spatially separated domains of *Fgfr3* expression are clearly visible (A). *Olig2* is expressed predominantly within the ventral 'gap' of *Fgfr3* expression (B). This suggests that pMN (bracketed), which generates *Pdgfra*+ oligodendrocyte progenitors (OLPs), does not also generate *Fgfr3*+ putative astrocyte progenitors. Scale bar: 50 μm.

Fig. 3. Incorporation of BrdU by *Fgfr3*-expressing cells. We labelled E18 embryos by two intra-peritoneal injections of BrdU, 6 hours apart, into the mother. We harvested the embryos 3 hours later and performed in situ hybridisation for *Fgfr3* followed by immunohistochemistry for BrdU. The *Fgfr3* (A) and BrdU (B) images were superimposed using Adobe Photoshop (C). Many *Fgfr3*-expressing cells incorporated BrdU (C, arrows), confirming that they can divide after exiting the VZ and are therefore unlikely to be neurones. Arrowheads in B,C indicate *Fgfr3*-negative cells that have incorporated BrdU.

after stage 34 (E8), both lateral and dorsal to the *Fgfr3*+ neuroepithelial domains. Often the individual cells appeared to be streaming away from the VZ into the parenchyma. This is evident in Fig. 1C, for example. By stage 37 (E11) *Fgfr3* expression was no longer detectable in the VZ but scattered *Fgfr3*+ cells were present throughout the grey and white matter of the cord (Fig. 1D). *Fgfr3* expression followed a similar progression in mouse and rat (Fig. 1F,G and not shown). In rodents, however, the ventral gap in *Fgfr3* expression was not as pronounced as in chicks (Fig. 1G, arrow).

A scattered population of *Fgfr3*-expressing cells is found throughout most regions of the late embryonic and postnatal mouse brain, both in white and in grey matter. As in the embryonic spinal cord, there appear to be specific regions of the embryonic brain VZ that give rise to *Fgfr3*+ cells that stream away from the VZ into the parenchyma (not shown).

Fgfr3-expressing cells originate mainly outside the pMN domain of the neuroepithelium

In the developing spinal cord, neuroepithelial precursors at different positions along the dorsoventral axis generate distinct neuronal subtypes. The ventral half of the spinal cord VZ is

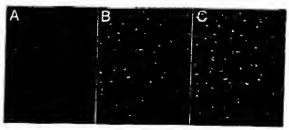

Fig. 4. Different populations of *Fgfr3*+ and *Pdgfra*+ cells in the newborn spinal cord. We hybridised sections of P2 mouse cervical spinal cord simultaneously with a DIG-labelled *Fgfr3* probe together with an FITC-labelled *Pdgfra* probe to visualise OLPs. The *Fgfr3* signal (red) was visualised with rhodamine-tyramide reagent and the *Pdgfra* signal (green) with fluorescein-tyramide. Scattered individual *Fgfr3*+ and *Pdgfra*+ cells can be seen throughout both white and grey matter of the cord, but these are separate and discrete cell populations. We conclude that the great majority of *Fgfr3*+ cells in the cord are not OLPs.

divided into five neuroepithelial domains known as (from ventral to dorsal) p3, pMN, p2, p1 and p0 (Briscoe et al., 2000). Of these, pMN is known to generate motoneurones followed by oligodendrocyte progenitors (OLPs). It seemed to us that the ventral gap in *Fgfr3* expression (Fig. 2A) might correspond to pMN. To test this, we performed double in situ hybridisation for *Fgfr3* and *Olig2* (which defines pMN) (Lu et al., 2000; Zhou et al., 2000). At stage 35, the *Olig2* in situ hybridisation signal was within the gap in the *Fgfr3* signal (Fig. 2B, arrow). Therefore, *Fgfr3* is preferentially downregulated in pMN where oligodendrocyte lineage cells originate, but is expressed both ventral and dorsal to pMN.

Fgfr3- expressing cells are glia

The fact that most of the scattered *Fgfr3*+ cells are generated after stage 34 (E8) in the chick, E13.5 in mouse, is itself a strong argument that they are glial cells, not neurones, because most spinal neurones are born before this (Altman and Bayer, 1984). That some of the *Fgfr3*+ cells are found in axon tracts also suggests that they are glia, for there are very few neuronal cell bodies in fibre tracts.

Another indication that they are glial cells is that they continue to divide after they leave the VZ. We showed this by injecting BrdU into a pregnant mouse at18 days gestation. The embryos were removed 3 hours later and processed by in situ hybridisation for *Fgfr3* followed by immunolabelling for BrdU. We found many (*Fgfr3*+, BrdU+) cells scattered throughout the white and grey matter of the cord (Fig. 3, arrows). This confirms that *Fgfr3*-expressing cells divide in vivo and are therefore unlikely to be neurones or neuronal progenitors, which leave the VZ as postmitotic cells. This strengthens the idea that the *Fgfr3*-expressing cells are glia. There was also a population of (BrdU+, *Fgfr3*-) cells in both grey and white matter (Fig. 3C, arrowheads), so there is a distinct population(s) of dividing cells that do not express *Fgfr3*.

Fgfr3-expressing cells are distinct from Pdgfra+ oligodendrocyte progenitors

To determine whether the *Fgfr3*+ cells that we detect are oligodendrocyte progenitors (OLPs), we double labelled

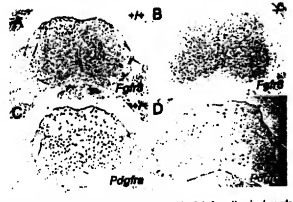

Fig. 5. *Fgfr3*-positive cells are unaffected in *Pdgfa* null spinal cords. Consecutive sections of newborn wild-type or *Pdgfa* knockout mouse cervical spinal cords were hybridised in situ with probes to *Fgfr3* (A,B) or *Pdgfra* (C,D). The number of *Pdgfra*$^+$ OLPs is strongly reduced in the *Pdgf-A* knockout (compare C with D) but neither the number nor the distribution of *Fgfr3*$^+$ cells is changed noticeably (A,B). Again, we conclude that the *Fgfr3*$^+$ cells and *Pdgfra*$^+$ OLPs are different cells.

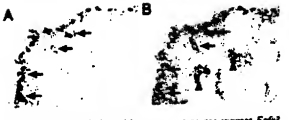

Fig. 6. Newly differentiating white matter astrocytes express *Fgfr3*. We simultaneously hybridised sections of E18 mouse cervical spinal cord with an FITC-labelled *Gfap* mRNA probe (A) and a DIG-labelled *Fgfr3* probe (B). The *Gfap* and *Fgfr3* hybridisation signals were visualised and photographed sequentially (see Materials and Methods). All the *Gfap*-expressing astrocytes also expressed *Fgfr3* (e.g. arrows). In general, *Fgfr3*$^+$ cells in the grey matter (arrowheads) did not co-express *Gfap*.

mouse E18 and P2 spinal cord sections for *Fgfr3* and *Pdgfra*, an established marker of early OLPs. Both in situ hybridisation probes labelled similar numbers of cells that were scattered throughout the spinal cord grey and white matter, but the two cell populations were completely non-overlapping (Fig. 4). This also held true throughout the postnatal brain (N. P. P., unpublished). We also looked in newborn *Pdgfa* knockout mice which contain far fewer *Pdgfra*$^+$ OLPs than normal (Fruttiger et al., 1999). Despite the lack of OLPs, there were normal numbers of *Fgfr3*$^+$ cells at this age (Fig. 5). Clearly, the *Fgfr3*$^+$ cells detected by our in situ hybridisation procedures are not early OLPs but a different cell population. This is consistent with the fact that in mice lacking Fgfr3, early events of oligodendrocyte lineage progression occur normally and the numbers of *Pdgfra*$^+$ cells remains unchanged (R. Bansal, personal communication) (N. P. P., unpublished).

Fgfr3-expressing cells are astrocytes and astrocyte precursors

To test whether the *Fgfr3*$^+$ cells might be astrocytes, we double labelled E18 mouse spinal cord sections for *Fgfr3* and *Gfap* mRNAs. At E18, white matter astrocytes begin to express *Gfap* mRNA, which initially remains in the astrocyte cell bodies and allows identification of individual astrocytes. (As astrocytes mature further, both *Gfap* mRNA and protein are relocated to the extending cell processes, making individual cells difficult to distinguish.)

All the *Gfap*$^+$ astrocytes in developing white matter at E18 also expressed *Fgfr3* (Fig. 6, arrows). This result clearly identifies many of the *Fgfr3*-expressing cells as astrocytes. Nevertheless, the majority of *Fgfr3*-expressing cells in the grey matter (Fig. 6B, arrowheads) are *Gfap*-negative. We presume that these represent *Gfap*-negative, possibly immature, astrocytes.

In an attempt to label all astrocytes, including *Gfap*-negative astrocytes, we used an in situ hybridisation probe against glutamine synthetase mRNA (*Glns*) (EC 6.3.1.2). Glns is widely regarded as an astrocyte marker, although there have been reports that it is also present in mature oligodendrocytes and even OLPs. We found that *Glns* transcripts were present in the VZ of the E15 mouse spinal cord and in cells outside the VZ in a pattern that was very similar that of *Fgfr3* (Fig. 7). This is consistent with the view that *Fgfr3* and *Glns* mark astrocytes and their precursors. This conclusion was further strengthened by studies of cultured astrocytes (see below).

Cultured astrocytes co-express Gfap and Fgfr3

When CNS cells are dissociated and placed in culture, astrocytes in the culture upregulate Gfap and are easily recognisable. We dissociated and cultured cells from E17 rat cervical spinal cord and labelled them by in situ hybridisation for *Fgfr3* and by immunocytochemistry for Gfap. Almost all of the Gfap$^+$ astrocytes also expressed *Fgfr3* (Table 1; Fig. 8, arrows). There was also a small population of flat, fibroblast-like *Fgfr3*$^+$ cells that did not express Gfap (Fig. 8, arrowheads). The number of these cells decreased with time in culture; at 3 days they were 6% of all cells, by 9 days less than 1% (Table 1). These (*Fgfr3*$^+$, Gfap$^-$) cells might be astrocyte precursors or immature astrocytes that have not yet upregulated Gfap. In any

Table 1. E17 rat spinal cord cell cultures double labelled for *Fgfr3* and Gfap

Days in vitro	*Fgfr3*$^+$ Gfap$^-$ (astrocyte precursors?)	*Fgfr3*$^+$ Gfap$^+$ (astrocytes)	*Fgfr3*$^-$ Gfap$^+$ (astrocytes)	*Fgfr3*$^-$ Gfap$^-$ (other cells)
3	17/275 (6%)	69/275 (25%)	None	189/275 (68%)
6	23/596 (4%)	82/596 (14%)	2/596 (<1%)	489/596 (82%)
9	4/645 (<1%)	197/645 (31%)	1/645 (<1%)	443/645 (69%)

Dissociated cells from E17 rat spinal cord were cultured for 3, 6 or 9 days and then subjected to in situ hybridization for *Fgfr3* mRNA followed by immunohistochemistry for Gfap protein. We counted astrocytes (*Fgfr3*$^+$, Gfap$^+$ and *Fgfr3*$^-$, Gfap$^+$), putative astrocyte precursors (*Fgfr3*$^+$, Gfap$^-$) and other unidentified cells (*Fgfr3*$^-$, GFAP$^-$). The great majority of GFAP-expressing astrocytes also expressed *Fgfr3*. These data are from a single representative experiment (duplicate coverslips); comparable results were obtained in two additional independent experiments.

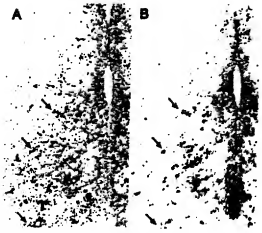

Fig. 7. Co-expression of *Fgfr3* and glutamine synthetase (*Glus*) in the VZ and parenchyma of the embryonic mouse spinal cord. There was considerable overlap between the in situ hybridisation signals for *Fgfr3* and *Glus* in the E15 mouse spinal cord, strongly suggesting that *Fgfr3*$^+$ cells correspond to glial (presumably astrocyte) precursors. Arrows indicate cells that express both *Fgfr3* and *Glus*.

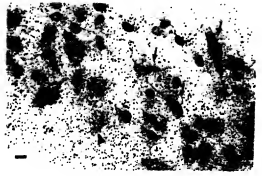

Fig. 8. Cultured cells from E17 rat spinal cord double-labelled for *Fgfr3* and Gfap. Cells were hybridised in situ with a ^{35}S-labelled RNA probe for *Fgfr3*, then immunolabelled for Gfap followed by autoradiography (see Materials and Methods). The *Fgfr3* signal (black silver grains) is present over most Gfap-positive cells (brown DAB reaction product; arrows) (also see Table 1). Scale bar: 10 μm. Arrowhead indicates an *Fgfr3*-positive, *Gfap*-negative cell.

case, this experiment provides clear evidence that most or all Gfap$^+$ astrocytes in culture co-express *Fgfr3*.

Gfap is upregulated in grey matter astrocytes in Fgfr3 null mice

If *Fgfr3* is expressed by astrocytes, we might expect to see specific effects on astrocytes in transgenic mice homozygous for a targeted disruption of *Fgfr3*. These mice have previously been shown to have skeletal and inner ear defects but no CNS defects have yet been reported (Colvin et al., 1996).

We visualised astrocytes in spinal cord sections of 3-month-old *Fgfr3* null mice, together with their heterozygous *Fgfr3*$^{+/-}$ and wild-type littermates, by immunolabelling with anti-Gfap. Heterozygous and null mutant mice all displayed the normal pattern of Gfap expression up to 6 weeks of age. Gfap expression was observed in the white matter around the circumference of the spinal cord, many Gfap-labelled processes being oriented in a radial direction (Fig. 9A). By comparison, there was little or no Gfap expression in the grey matter, except in astrocytes associated with blood vessels. Between 6 weeks and 2 months of age, a striking up-regulation of Gfap expression occurred in the grey matter of *Fgfr3* null mice, though not in their heterozygous or wild-type littermates (Fig. 9B). Astrocytes lining blood vessels also had increased Gfap immunoreactivity.

The number of cells that contain *Fgfr3* mRNA was not noticeably different in *Fgfr3*-null spinal cords compared with wild type (data not shown). This suggests that Fgfr3 does not normally mediate a signal for proliferation or survival of astrocytes, although further experiments (e.g. BrdU labelling in vivo) would be required to substantiate this.

Astrocyte development in vitro does not depend on Hedgehog signalling

In the spinal cord, production of ventral cell types – motoneurones, ventral interneurones and OLPs – is dependent on Shh signalling (Ericson et al., 1996; Orentas et al., 1999) (for a review, see Jessell, 2001). We wanted to know whether production of astrocytes from the ventral neural tube is also dependent on Shh. We microdissected stage 12/13 (E2) chick spinal cord into thirds along the dorsoventral axis and cultured the ventral-most fragments in collagen gels with either a control antibody or an anti-Shh neutralising antibody (see Materials and Methods). After 48 hours in culture we labelled explants with monoclonal antibody 4D5, which recognises homeodomain proteins Isl1 and Isl2 in motoneurones. Control explants contained numerous Isl$^+$ cells, whereas none were observed in explants incubated with anti-Shh (data not shown). After a further 10 days in culture (12 days total) we visualised OLPs with monoclonal antibody O4 (Sommer and Schachner, 1981) (Fig. 10C,D) and astrocytes with anti-Gfap (Fig. 10A,B). All of the explants incubated with control antibody (19/19) contained large numbers (>300) of O4$^+$ late-stage OLPs (Fig. 10C). As expected, OLP production was markedly decreased by anti-Shh (Fig. 10D); 14/22 explants contained no O4$^+$ cells and, of the remaining eight explants, seven contained fewer than ten positive cells and the other one contained 38 positive cells. By contrast, all explants contained numerous (>300) Gfap$^+$ astrocytes whether they had been incubated with control antibody (19/19) or anti-Shh (22/22) (Fig. 10A,B).

Similar results were obtained with explants from stage 25 (E5) embryos from which we were able to dissect the ventral one-quarter of the neural tube and discard the floor plate. Once again, large numbers of Gfap$^+$ astrocytes developed in explants cultured with control antibody (22/22) and with anti-Shh (25/25), even though OLP production in these explants was inhibited by anti-Shh (not shown).

To test the possibility that other hedgehog (Hh) proteins (Desert Hh, Indian Hh) control astrocyte production in ventral explants, we inhibited the activity of all isoforms with the alkaloid cyclopamine (Cooper et al., 1998; Incardona et al., 1998). This gave similar results as Shh neutralising antibodies (data not shown). Thus, we conclude that astrocyte induction in ventral spinal cord does not require Hedgehog signalling.

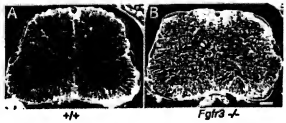

Fig. 9. Gfap upregulation in grey matter astrocytes in *Fgfr3*-null mice. Transverse sections through the cervical spinal cords of 2-month-old wild-type (A) and *Fgfr3*-null mice (B) were immunolabeled with anti-Gfap. In the wild-type cord, white matter (fibrous) astrocytes express Gfap but there is little or no Gfap immunoreactivity in the grey matter. By contrast, the *Fgfr3*-null mouse (B) shows extensive Gfap labelling of grey matter (protoplasmic) astrocytes. Scale bar: 100 μm.

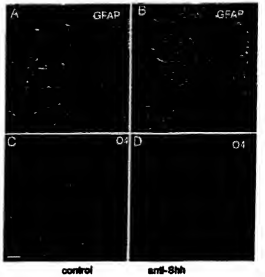

Fig. 10. Neutralising *Shh* activity in explant cultures of ventral spinal cord. Stage 12/13 (E2) chick neural tube was dissected into dorsal, intermediate and ventral thirds. The ventral thirds were cultured in collagen gels in the presence of either control antibody (A,C) or with neutralising anti-Shh antibody (B,D). Explants were double-labelled with O4 monoclonal antibody (C,D) and anti-Gfap (A,B). Anti-Shh blocks the formation of O4-positive OLPs but not Gfap-positive astrocytes. Scale bar: 10 μm.

DISCUSSION

On the basis of their spatial distribution and time of appearance, Peters et al. (Peters et al., 1993) suggested that *Fgfr3*$^+$ cells in the mouse CNS are glial cells, possibly astrocytes. By double labelling experiments with *Fgfr3* and Gfap, Miyake et al. (Miyake et al., 1996) concluded that *Fgfr3* was expressed in astrocytes in the adult rat brain. Our data support and extend these conclusions. We present evidence that scattered *Fgfr3*$^+$ cells in the embryonic and postnatal CNS are astrocytes and/or astrocyte progenitors, and that these astrocytes are derived from *Fgfr3*$^+$ neuroepithelial precursors in the VZ.

Fgfr3 is also expressed transiently by a subpopulation of motoneurones (Philippe et al., 1998) and by late oligodendrocyte progenitors (late OLPs) just prior to terminal differentiation in vitro (Bansal et al., 1996). We are convinced that the *Fgfr3*$^+$ cells that we detect are not OLPs, however. First and foremost, double labelling for *Fgfr3* and *Pdgfra* (a marker of early OLPs) demonstrates that these mark separate populations of cells. The *Fgfr3*$^+$ and *Pdgfra*$^+$ cell populations appear at different times and initially their distributions are different. Moreover, the number and distribution of *Fgfr3*$^+$ cells was unaltered in neonatal *Pdgfa*-null spinal cords, which have very few *Pdgfra*$^+$ OLPs and oligodendrocytes (Fruttiger et al., 1999). This argues strongly that the large majority of *Fgfr3*$^+$ cells revealed by our in situ hybridisation protocol are not OLPs. Bansal et al. (Bansal et al., 1996) have shown that OLPs do express *Fgfr3* mRNA in culture but only at a low level during the earlier stages of the lineage. Presumably this is below our limit of detection in situ. OLPs upregulate *Fgfr3* strongly just prior to oligodendrocyte differentiation (Bansal et al., 1996) but these presumably represent a small subset of OLPs in the embryonic spinal cord and do not feature in our analysis.

Fgfr3-positive cells co-expressed mRNA encoding glutamine synthetase (Glns; EC 6.3.1.2). In the CNS, Glns is an accepted marker of mature astrocytes (Norenberg and Martinez-Hernandez, 1979; Stanimirovic et al., 1999) but it is also expressed in oligodendrocytes (Domercq et al., 1999) and OLPS (Baas et al., 1998). Glns has not previously been ascribed to neurocepithelial precursors or immature astrocytes in the embryo, although *Glns* transcripts have been detected in the rat brain by northern blot as early as E14. To our knowledge, Glns has not been described in neurones except in pathological situations such as Alzheimer's disease (Robinson, 2000). Therefore, we are confident that the (*Fgfr3*$^+$, *Glns*$^+$) double-positive cells described here are glial cells. Taken together with the evidence against them being OLPs (see above), it seems likely that they correspond to immature and mature astrocytes. This is strongly supported by the observations that *Fgfr3*$^+$ cells co-express Gfap protein and/or mRNA in (1) the formative white matter of the normal developing spinal cord and (2) cultures of dissociated spinal cord cells.

Neuroepithelial origins of astrocytes

Fgfr3 was expressed in two domains of the spinal cord neuroepithelium separated by an *Fgfr3*-negative region. This was true of both rodent and avian embryos though it was more obvious in the latter. The *Fgfr3*-negative region corresponds roughly to the pMN domain of the VZ that generates somatic motoneurones followed by *Pdgfra*$^+$ OLPs (Sun et al., 1998; Rowitch et al., 2002). Therefore, our data indicate that OLPs and astrocytes originate from separate precursors that reside in different parts of the VZ. How does this fit with other ideas about the origin of astrocytes? One hypothesis is that astrocytes arise by transdifferentiation of radial glia, after the latter have fulfilled their role as cellular substrates for radial migration of neuronal progenitors (Bignami and Dahl, 1974; Choi et al., 1983; Benjelloun-Touimi et al., 1985; Voigt, 1989; Culican et al., 1990). This could be compatible with our *Fgfr3* expression data, as radial glia have their cell bodies close to the ventricular

surface. However, radial glia are distributed all around the spinal cord lumen, unlike *Fgfr3*, so one would have to postulate that only a subset of radial glia express *Fgfr3*.

In double-knockout mice that lack the two basic helix-loop-helix (bHLH) transcriptions factors Olig1 and Olig2, the pMN domain of the VZ undergoes a homeotic transformation into p2, its immediate dorsal neighbour (Rowitch et al., 2002). As a result, pMN no longer generates motoneurones followed by OLPs, but instead produces V2 interneurones followed by astrocytes (Zhou and Anderson, 2002; Takebayashi et al., 2002). By implication, this is the usual fate of p2 precursors in wild-type mice. This is consistent with our observation that *Fgfr3*+ astrocytes apparently originate within an extended part of the ventral VZ, including p2 but excluding pMN. Our *Fgfr3* expression data are also consistent with previous fate mapping experiments in chick-quail chimeras, which indicated that astrocytes are generated from dorsal as well as ventral parts of the VZ, whereas OLPs are generated only from ventral territory (Pringle et al., 1998). It remains to be seen whether astrocytes that are generated from distinct neuroepithelial domains (p3 or p2, say) have identical properties or whether they are functionally specialised – for modulating synaptic activity or interacting with blood vessels, for example.

Production of ventral cell types such as motoneurones, interneurones and OLPs is dependent on Shh signalling. As many *Fgfr3*-expressing astrocyte precursors appear to originate in p3, p2 and other ventral domains, we might expect that production of astrocytes might also depend on Shh signalling. However, we found that astrocytes developed in explant cultures of ventral neural tube either in the presence or absence of Shh activity. Our data imply that astrocytes are specified by different mechanisms than OLPs – at least, they demonstrate that astrocyte and OLP production are not obligatorily linked. In fact, there is evidence that more than one signalling pathway can lead to astrocyte development in vitro (Rajan and McKay, 1998). Because astrocytes can be formed from dorsal as well as ventral neuroepithelium, it remains possible that 'ventral' astrocytes might normally be under Shh control, but that by blocking Shh signalling we uncover an alternative 'dorsal' pathway for astrocyte development.

It has been reported that there are glial-restricted precursor cells (GRPs) in the embryonic rat spinal cord that are dedicated to the production of astrocytes and oligodendrocytes (Rao and Mayer-Proschel, 1997; Herrera et al., 2001). This seems to conflict with current evidence that oligodendrocytes and astrocytes are generated from different precursors in the embryonic spinal cord (Lu et al., 2002; Rowitch et al., 2002; Zhou and Anderson, 2002) (this paper). A possible reconciliation might be that GRPs with the potential to generate both astrocytes and oligodendrocytes are formed in all parts of the spinal cord VZ but are constrained in vivo to generate only astrocytes or only oligodendrocytes, depending on the signals in their local environment (i.e. where they are located) (for a review, see Rowitch et al., 2002).

Fgfr3 regulates Gfap expression in grey matter astrocytes

Astrocytes with distinct, heritable morphologies have been described in cultures of rat spinal cord cells (Fok-Seang and Miller, 1992). Astrocytes in different parts of the CNS differ in morphology or function in vivo too, suggesting that they might fulfil different, region-specific functions. In addition, astrocytes in white matter tracts generally have smaller cell bodies with more and longer processes compared to their counterparts in grey matter (Connor and Berkowitz, 1985). For this reason, white matter astrocytes are sometimes referred to as 'fibrous' and those in grey matter as 'protoplasmic' or 'velous'. White matter astrocytes also express high levels of Gfap, whereas grey matter astrocytes contain little or no immunoreactive Gfap.

Fibrous and protoplasmic astrocytes might develop from separate lineages (Connor and Berkowitz, 1985). However, our observation that Gfap is upregulated in grey matter astrocytes of *Fgfr3*-null mice provides strong in vivo evidence that extracellular signals might be required to maintain their normal Gfap-negative phenotype. This is consistent with a report that adding Fgf2 to cultured astrocytes downregulates Gfap mRNA and protein and causes their morphology to change (Reilly et al., 1998). Fgf2 and other known Fgfr3 ligands such as Fgf9 are made by, and presumably released from, many CNS neurones (Eckenstein et al., 1991; Cotman and Gomez-Pinilla, 1991; Woodward et al., 1992; Gomez-Pinilla et al., 1994; Kuzis et al., 1995). One possible reason that white matter astrocytes express high levels of Gfap in wild-type mice might be that they are denied exposure to *Fgfr3* ligands in axon tracts – perhaps because Fgf, like Pdgf, is secreted from neuronal cell bodies but not from axons (Fruttiger et al., 2000).

Upregulation of Gfap in the *Fgfr3*-null mouse is mindful of the astrocyte response to CNS injury or disease – so-called reactive gliosis or astrocytosis (for reviews, see Ridet et al., 1997; Norton, 1999). It would be interesting to know whether interruption of signalling through Fgfr3 is somehow involved in the astrocyte reaction to injury. However, it is unlikely to be straightforward, because Gfap upregulation in the *Fgfr3*-null animals does not occur until around 2 months of age, suggesting that it is an indirect effect. In addition, the data from the *Fgfr3*-null mouse are difficult to square with the observation that intra-ventricular injection of Fgf2 has been reported to increase the number of Gfap+ reactive astrocytes (Unsicker, 1993).

Most grey matter (protoplasmic) astrocytes possess many short sheet-like processes containing little, if any, Gfap (Connor and Berkowitz, 1985). It has been suggested that this morphology might help them to infiltrate the neuropil and surround axonal terminals, synapses and neuronal cell bodies, consistent with one of their proposed roles in neurotransmitter metabolism (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979). It will be interesting to see if the reactive astrocytes in *Fgfr3*-null mice are defective in neurotransmitter metabolism and whether this contributes to the premature death of the animals.

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EVIDENCE APPENDIX
EXHIBIT 13

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A stochastic model of brain cell differentiation in tissue culture

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Abstract. The timing of cell differentiation can be controlled both by cell-intrinsic mechanisms and by cell-extrinsic signals. Oligodendrocyte type-2 astrocyte progenitor cells are known to be the precursor cells that give rise to oligodendrocytes. When stimulated to divide by purified cortical astrocytes or by platelet-derived growth factor, these progenitor cells generate oligodendrocytes *in vitro* with a timing like that observed *in vivo*. The most widely accepted model of this process assumes a cell-intrinsic biological clock that resides in the progenitor cell. The intrinsic clock model originally proposed in 1986 remains as the dominant theoretical concept for the analysis of timed differentiation in this cell lineage. However, the results of a recent experimental study (Ibarrola et al., Developmental Biology, vol. 180, 1–21, 1996) are most consistent with the hypothesis that the propensity of a clone of dividing O-2A progenitor cells initially to generate at least one oligodendrocyte may be regulated by cell-intrinsic mechanisms, but that environmental signals regulate the extent of further oligodendrocyte generation. We propose a stochastic model of cell differentiation in culture to accommodate the most recent experimental findings. Our model is an age-dependent branching stochastic process with two types of cells. The model makes it possible to derive analytical expressions for the expected number of progenitor cells and of oligodendrocytes as functions of time. The model parameters were estimated by fitting these functions through data on the average (sample mean) number of both types of cells per colony at different time intervals from start of experiment. Using this method we provide a biologically meaningful interpretation of the observed pattern of oligodendrocyte generation *in vitro* and its modification in the presence of thyroid hormone.

Key words: Brain cells – Differentiation – Proliferation – Branching process

1 Introduction

It is a striking feature of ontogenic development that particular cell types first appear at precisely regulated moments in the history of the organism and then increase in number over a time period that is very similar in members of the same species. Understanding the biological principles which underly such appropriately timed cell generation is one of the profound challenges in developmental biology.

Any analysis of the timely generation of differentiated cell types must by necessity begin with investigation of the transition from dividing precursor cell to differentiated (and often non-dividing) progeny cell. In order to obtain data that allow one to develop an understanding of the complexities of such differentiation processes, it is necessary to gain access to a wide range of detailed information at a clonal level. Ideally, one should be able to examine multiple clones of dividing precursor cells and to unambiguously distinguish between precursor cells and differentiated progeny. If one is interested in analysis of these processes in cells derived from vertebrates, then experimental studies of this nature must be performed in tissue culture (to enable visualization of cell clones and their development over extended time periods), leading to the further requirement that it be possible to mimic *in vitro* the developmental processes which are thought to occur *in vivo*.

One of the few cellular lineages in which it is possible to obtain the complete collection of data described above is derived from the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell of the central nervous system. It is possible to grow O-2A progenitors cells *in vitro* in such a manner that they divide and generate oligodendrocytes with a timing which recapitulates the timing of normal development *in vivo*. O-2A progenitor cell division can be promoted by purified cortical astrocytes (of a separate glial lineage), and this induction of cell division can also be brought about by platelet-derived growth factor (PDGF) and by the O-2A progenitor mitogen produced by cortical astrocytes (Noble and Murray, 1984; Noble et al., 1988; Pringle et al., 1991; Raff et al., 1988; Bogler et al., 1990). Embryo-derived O-2A progenitor cells induced to divide by PDGF or by type-1 astrocytes will generate oligodendrocytes with a timing that mimics *in vitro* the timing which is seen *in vivo* (Raff et al., 1988).

The initiation of the timely generation of oligodendrocytes within a clonal family of dividing O-2A progenitor cells has for many years been thought to be controlled by a cell-intrinsic biological clock that induces symmetric and synchronous differentiation of all clonally-related O-2A progenitor cells into oligodendrocytes within a relatively short period of time (Temple and Raff, 1985). According to this model, oligodendrocyte generation is associated with preclusion of the self-renewal process of precursor cells, and thus is in some ways analogous to the limited mitotic life-span expressed by many primary cell types. This hypothesis provided a simple model for the study of temporally regulated differentiation, but it now seems likely that this hypothesis is both incorrect and overly simplistic.

In contrast to the above hypothesis, it was recently discovered that instead of differentiating symmetrically, clonal families of O-2A progenitor may undergo a protracted period of oligodendrocyte generation, during which time the probability of precursor cell self-renewal is regulated predominantly by cell-extrinsic signaling molecules rather than by cell-intrinsic biological clocks (Ibarrola et al., 1996). In other words, it was found that it is possible to distinguish experimentally between the probability that a clone of dividing O-2A progenitor cells will generate at least one oligodendrocyte at an appropriate time *in vitro* and the actual extent of oligodendrocyte generation in that clone. While the latter process was influenced by the presence or absence of exogenous factors (such as thyroid hormone), the former was not.

In respect to the initial generation of oligodendrocytes, it appears that the extent of oligodendrocyte generation within an individual clone may be stochastic. For example, there was no apparent relationship between the number of oligodendrocytes found and the ratio of oligodendrocytes to progenitor cells, excepting that the rare colonies consisting wholly of oligodendrocytes all contained 10 cells or less. In addition, when oligodendrocytes first appeared in cultures of embryonic brain cells the number of oligodendrocytes per colony ranged from 1 to 88, the proportion of oligodendrocytes in heterogeneous colonies ranged from <1% to 81%, and the fractional representation of oligodendrocytes in a colony was not correlated with the number of oligodendrocytes in that colony.

In this paper, we develop a stochastic model which is specifically designed to make quantitative inferences from experimental data on brain cell differentiation *in vitro*.

2 Experimental procedures

Purification of O-2A progenitor cells is described at length by Ibarrola et al. (1996). Purified progenitor cells derived from 7 day old animals were plated at a density of 2500-3000 cells. Cells were fed with PDGF. After plating the culture was scored for the presence of individual cells. Plates with cells in clumps were discarded and not included in the experiment. A fixed number of clones was randomly selected and the cell type composition of each clone was recorded at different times. Cell-types were identified by morphology and cell types were confirmed at the end of each experiment by immunofluorescence using cell type specific antibodies. The design of experiments with thyroid hormone was identical.

3 The model for oligodendrocyte generation in culture

Our model for the stochastic mechanism of O-2A progenitor cell differentiation *in vitro* is a multitype age-dependent branching stochastic process. The model structure is defined by the following set of assumptions.

(i) The process begins with a single progenitor, or type-1, cell cultured at time $t = 0$. This initiator cell may, when it divides, produce two types of cells: progenitor cells of the same type, and oligodendrocytes or type-2 cells. The initiator cell and its descendants are not susceptible to death.

(ii) At the end of the mitotic cycle, every type-1 cell gives rise to two daughter progenitor cells with probability p, and it transforms (differentiates) into an oligodendrocyte with probability $1 - p$. Thus, the probability generating function of the cell progeny is specified as

$$h_1(s) = ps_1^2 + (1 - p)s_2 , \tag{1}$$

where $s = (s_1, s_2)$. The corresponding generating function for type-2 cells is

$$h_2(s) = s_2 , \tag{2}$$

that is, oligodendrocytes neither divide nor die. We do not provide a description of cell death, lest the model be nonidentifiable. The event of cell death is very rare during the first 6 days of observation (Ibarrola et al., 1996), and we take advantage of this fact when estimating the model parameters. The death of oligodendrocytes normally begins on day 7 and its rate increases with time. The contribution of cell death at these late times can be inferred from experimental data by the indirect route.

(iii) The lengths of the mitotic cycle of the initiator cell and its descendants of the same type are independent and identically distributed nonnegative random variables with a common cumulative distribution function $F(t)$. We assume that $F(0) = 0$.

(iv) Progenitor cells, the only migratory cells in the population, do not migrate out of the field of observation.

(v) The usual independence assumptions regarding the evolution of age-dependent branching processes (Jagers, 1975) are adopted.

The model thus specified is a special case of the Bellman-Harris branching process with two types of particles, its asymptotic properties were studied in detail by Jagers (1969, 1975).

Let $Z_i(t)$, $i = 1, 2$, be the number of cells of the ith type at time t. The probability generating functions of $Z_1(t)$ and $Z_2(t)$ can be obtained using general methods of the theory of branching processes. A detailed exposition of these methods can be found in Yakovlev and Yanev (1989). Consider the two-dimentional stochastic process $Z(t) = (Z_1(t), Z_2(t))$ and introduce the generating function

$$\Phi(t, s) = (\Phi_1(t, s), \Phi_2(t, s)) , \tag{3}$$

with the components

$$\Phi_i(t, s) = \sum_k p_{ik}(t|Z(0) = e_i)s^k , \quad i = 1, 2, |s| \le 1 , \tag{4}$$

where $e_1 = (1, 0)$, $e_2 = (0, 1)$, and the summation in (4) is over the set of all points in R^2 with nonnegative integer coordinates. With $h_1(s)$ and $h_2(s)$ given by (1) and (2), the generating functions $\Phi(t, s)$ satisfy the following equations

$$\Phi_1(t, s) = s_1[1 - F(t)] + p\int_0^t \Phi_1^2(t - u, s)\,dF(u) + (1 - p)\int_0^t \Phi_2(t - u, s)\,dF(u), \tag{5}$$

$$\Phi_2(t, s) = s_2 .$$

Setting $s_1 = z$ and $s_2 = 1$ in (5), it is easy to derive equations for the corresponding marginal generating functions $\varphi_1(t, z) = \Phi_1(t, z, 1)$ and $\varphi_2(t, z) = \Phi_2(t, z, 1)$:

$$\varphi_1(t, z) = z[1 - F(t)] + (1 - p)F(t) + p\int_0^t \varphi_1^2(t - u, z)\,dF(u), \qquad \varphi_2(t, z) = 1 . \tag{6}$$

In a similar manner, for $\psi_1(t, z) = \Phi_1(t, 1, z)$ and $\psi_2(t, z) = \Phi_2(t, 1, z)$ we obtain

$$\psi_1(t, z) = 1 - F(t) + p\int_0^t \psi_1^2(t - u, z)\,dF(u) + (1 - p)zF(t), \qquad \psi_2(t, z) = z . \tag{7}$$

Let $M_1(t) = \varphi_1'(t, 1)$ be the expected number of type-1 cells at time t. From (6) it follows that

$$M_1(t) = 1 - F(t) + 2p\int_0^t M_1(t - u)\,dF(u) . \tag{8}$$

The expected number of type-2 cells is $M_2(t) = \psi_2'(t, 1)$. Thus we see from (7) that

$$M_2(t) = (1 - p)F(t) + 2p\int_0^t M_2(t - u)\,dF(u) \tag{9}$$

The integral equations (8) and (9) can be generalized to incorporate possible death of type-1 cells (Jagers, 1975). Furthermore they can be solved in closed form (see Athreya and Ney, 1972). Introducing the notation

$$G^{*0}(t) = 1, \qquad G^{*1}(t) = G(t), \qquad G^{*(n+1)}(t) = \int_0^t G^{*n}(t - u)\,dG(u) ,$$

the solution of (8) is represented as

$$M_1(t) = \sum_{n=0}^{\infty} (2p)^n[F^{*n}(t) - F^{*(n+1)}(t)] \tag{10}$$

Similarly the solution of (9) is given by

$$M_2(t) = (1 - p)\sum_{n=0}^{\infty} (2p)^n F^{*(n+1)}(t) \tag{11}$$

The n-fold convolution F^{*n} of F with itself can be found in an explicit form for some distributions of the mitotic cycle duration. The most popular choice in cell-kinetics studies is the two-parameter gamma distribution (Yakovlev et al., 1977; Nedelman and Rubinow, 1981; Nedelman et al., 1987; Yakovlev and Yanev, 1989, to name a few). We shall proceed from the same choice because this parametric family is quite flexible and reflects a multistage structure of the cell cycle. Some authors (Hartmann et al., 1975; Yakovlev and Zorin, 1988) indicate that the results of kinetic analysis of cell proliferation are usually insensitive to the form of $F(t)$ where absolutely continuous unimodal distributions are concerned.

Suppose that $F(t)$ is specified as the gamma distribution with shape parameter α and scale parameter β. Then we have

$$F^{*n}(t) = \frac{\beta^{\alpha n}}{\Gamma(\alpha n)} \int_0^t x^{\alpha n - 1} e^{-\beta x} dx . \tag{12}$$

To ensure computationally convenient formulas for $M_1(t)$ and $M_2(t)$ it is reasonable to limit possible values of α to the set of positive integers. This constraint has little effect on the accuracy of estimation of the parameter α from experimental data on the mean size of cell clones. For integer α formula (12) reduces to

$$F^{*n}(t) = 1 - e^{-\beta t} \sum_{k=0}^{\alpha n - 1} \frac{(\beta t)^k}{k!} , \tag{13}$$

and we have

$$M_1(t) = e^{-\beta t} \left\{ \sum_{k=0}^{\alpha - 1} \frac{(\beta t)^k}{k!} + \sum_{n=1}^{\infty} (2p)^n \left[\sum_{k=0}^{\alpha n + \alpha - 1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{\alpha n - 1} \frac{(\beta t)^k}{k!} \right] \right\} , \tag{14}$$

$$M_2(t) = (1 - p) \left[\frac{1}{1 - 2p} - e^{-\beta t} \sum_{k=0}^{\alpha - 1} \frac{(\beta t)^k}{k!} - e^{-\beta t} \sum_{n=1}^{\infty} (2p)^n \sum_{k=0}^{\alpha n + \alpha - 1} \frac{(\beta t)^k}{k!} \right] . \tag{15}$$

The timing of oligodendrocyte generation *in vitro* has been found to be fundamentally similar to that which occurs *in vivo*. Therefore, it is natural to assume that the population of progenitor cells eventually becomes extinct. In terms of our model this means that the process $Z_t(t)$ is subcritical and we should limit our consideration to the case $p < 0.5$. Then formula (11) implies that $M_2(t)$ is a monotone nondecreasing function and

$$M_2(0) = 0, \qquad \lim_{t \to \infty} M_2(t) = \frac{1 - p}{1 - 2p} . \tag{16}$$

The behavior of $M_2(t)$ is intuitively appealing because in the long run the population of oligodendrocytes is expected to level off to a constant size.

Difficulties emerge when we look more closely at the expected number of progenitor cells. Recalling formula (10) we see that

$$M_1(0) = 1, \qquad \lim_{t \to \infty} M_1(t) = 0 \tag{17}$$

It also follows from (10) that $M_1(t)$ is a nonincreasing function of time. Indeed, representing the series (10) as

$$M_1(t) = \sum_{n=0}^{\infty} (2p)^n F^{*n}(t) - \sum_{n=0}^{\infty} (2p)^n F^{*(n+1)}(t)$$

$$= \sum_{n=0}^{\infty} (2p)^n F^{*n}(t) - F(t) - \sum_{n=0}^{\infty} (2p)^{n+1} F^{*(n+2)}(t)$$

$$= 1 - (1-2p)F(t) + \sum_{n=2}^{\infty} (2p)^n F^{*n}(t) - \frac{1}{2p} \sum_{n=0}^{\infty} (2p)^{n+2} F^{*(n+2)}(t)$$

$$= 1 - (1-2p)\left[F(t) + \frac{1}{2p} \sum_{n=2}^{\infty} (2p)^n F^{*n}(t) \right],$$

it is easy to see that $M_1(t)$ is nonincreasing in t whenever $p < 0.5$.

The behavior of $M_1(t)$ appears to be in conflict with experimental data presented in Sect. 4. Since each colony begins with exactly one clonogenic cell at time $t = 0$ one should expect (see Figs. 1 and 2, Sect. 4) that the growth curve for progenitor cells passes through a maximum before it starts decreasing; this is the only pattern consistent with our observations. Clearly, the model must be generalized to allow for the initial increase in the mean number of type-1 cells. The way to do this is through assigning a higher (greater than 0.5) probability of progenitor cell division to initial mitotic cycles. To keep the number of unknown parameters to a minimum, we assume that $p = 1$ for the first N cycles and $p < 0.5$ for the subsequent mitotic cycles. In other words, a progenitor cell acquires the competence for differentiation only after it undergoes a critical number of mitotic divisions. The parameter N is to be estimated from experimental data.

The stepwise change of the probability p can be readily incorporated into the model by introducing N additional types of cells that correspond to the N initial mitotic cycles. We will omit the derivation of the expressions for $M_1(t)$ and $M_2(t)$ given below because it parallels that of formulas (14) and (15). The functions $M_1(t)$ and $M_2(t)$ are given by

$$M_1(t) = e^{-\beta t}\left\{ \sum_{k=0}^{N-1} \frac{(\beta t)^k}{k!} + \sum_{j=1}^{N-1} 2^j \left(\sum_{k=0}^{n(N+1)-1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{nj-1} \frac{(\beta t)^k}{k!} \right) \right.$$
$$+ 2^N \left[\sum_{k=0}^{n(N+1)-1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{nN-1} \frac{(\beta t)^k}{k!} \right.$$
$$\left. \left. + \sum_{n=1}^{\infty} (2p)^n \left(\sum_{k=0}^{n(N+n+1)-1} \frac{(\beta t)^k}{k!} - \sum_{k=0}^{n(N+n)-1} \frac{(\beta t)^k}{k!} \right) \right] \right\}, \tag{18}$$

$$M_2(t) = 2^n(1-p)\left[\frac{1}{1-2p} - e^{-\beta t} \sum_{k=0}^{n(N+1)-1} \frac{(\beta t)^k}{k!} \right.$$
$$\left. - e^{-\beta t} \sum_{n=1}^{\infty} (2p)^n \sum_{k=0}^{n(N+n+1)-1} \frac{(\beta t)^k}{k!} \right]. \tag{19}$$

where $N = 1, 2, \ldots, \sum_{k=1}^{\infty} \chi_k = 0$, and $0 < p < 0.5$. It immediately follows from (18) and (19) that $M_1(t)$ still satisfies (17) while $M_2(t)$ has the following properties:

$$M_2(0) = 0, \qquad \lim_{t \to \infty} M_2(t) = 2^N \frac{1-p}{1-2p}. \tag{20}$$

4 Inference from experimental data

Equations (19) and (20) provide the basis for estimation of the model parameters by fitting the functions $M_1(t)$ and $M_2(t)$ through data on the average (sample mean) number of both types of cells per colony at different time intervals from start of experiment. We used the least squares method for this purpose. To minimize the sum of squared residuals, use was made of the flexible simplex method (Himmelblau, 1972). The results produced by this nonlinear programming procedure were verified with a program for function minimization included in *MATHEMATICA*.

At a time when we conducted our analysis only the data for $t = 72, 96, 120$ and 144 hours were available, furnishing an opportunity to test the predictive power of the model. In what follows we will refer to these data as Experiment 1. Using the data from Experiment 1 we obtained the following estimates of the model parameters: $\hat{p} = 0.461$, $\hat{N} = 2$, $\hat{\delta} = 3$, $\hat{\beta} = 0.107$. Thus, the mean, $\tau = \alpha/\beta$, and the standard deviation, $\sigma = \sqrt{\alpha}/\beta$, of the mitotic cycle duration are estimated as $\hat{\tau} = 28$ h and $\hat{\sigma} = 16.2$ h, respectively. The resultant fit is shown in Fig. 1. The model predicts that the mean number of oligodendrocytes tends to a constant level of 27.6 (see formula (20)). Unfortunately, testing this prediction cannot be carried out without the addition of oligodendrocyte survival factors (Noble, 1997), all of which are known to themselves affect the probability of cell differentiation. In our experiments, the cells do not survive that long.

A similar analysis of oligodendrocyte generation in the presence of thyroid hormone (Fig. 2) resulted in the following estimates: $\hat{p} = 0.287$, $\hat{N} = 2$, $\hat{\delta} = 3$, $\hat{\beta} = 0.137$, whence we have $\hat{\tau} = 21.3$ h and $\hat{\sigma} = 12.6$ h. Hence, thyroid hormone exerts a twofold effect on the cell system under study; it reduces the mean duration of the mitotic cycle of pregenitor cells, and it increases the probability of their transformation into oligodendrocytes. The observed dynamics of accumulation of oligodendrocytes is attributable to these mechanisms. It is seen from Fig. 2 that in the presence of thyroid hormone the mean size of the population of oligodendrocytes grows more rapidly attaining a much lower constant level of 6.7 cells predicted by formula (20). At the same time the critical number of initial cycles, N, remains unaltered. These results are in full agreement with qualitative conclusions made by Ibarrola et al. (1996).

An independent experimental study (Experiment 2) was conducted to test the model behavior beyond the observation period chosen in Experiment 1. In

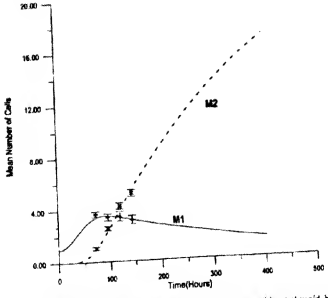

Fig. 1. The dynamic behavior of cell populations in cultures without thyroid hormone (Experiment 1). Solid line represents the expected number of progenitor cells as a function of time; dashed line represents the expected number of oligodendrocytes; + and × are the corresponding experimental data. Bars represent one root square error of estimation at each time point

Experiment 2, data were recorded at $t = 48$, 144 and 192 hours and compared with the corresponding values of $M_1(t)$ and $M_2(t)$ resulted from the analysis of Experiment 1. These results are summarized in Tables 1–4. It is clear from these tables that the model provides a good description of Experiment 2 except the mean number of oligodedrocytes at 192 h (Tables 2 and 4). The observed discrepancy can be attributed to a high rate of cell death at this late time which is not incorporated into the model. As our independent observations show, the proportion of oligodendrocytes surviving by day 8 under *in vitro* conditions is no more than 80%. Yet another factor that may contribute to the discrepancy under discussion is the inter-experimental variation which is difficult to control because the experiments of this kind are very time consuming. One can see from the data for $t = 144$ h that the mean number of oligodendrocyes tends to be smaller in Experiment 2 than in Experiment 1. This tendency may be a manifestation of the higher rate of cell death in Experiment 2 indicated above. However, there is little point in incorporating an explicit description of the process of cell death into the model because the

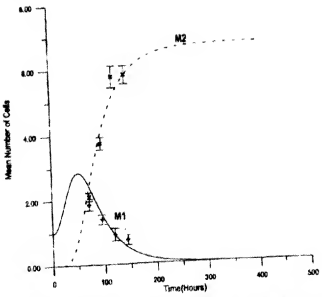

Fig. 2. The dynamic behavior of cell populations in cultures with added thyroid hormone (Experiment 1). Solid line represents the expected number of progenitor cells as a function of time, dashed line represents the expected number of oligodendrocytes. + and × are the corresponding experimental data. Bars represent one root square error at each time point.

Table 1. Mean number of O-2A progenitor cells in the absence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_1(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
48	2.68	–	3.4	–	0.24
72	3.40	3.67	–	0.20	–
96	3.54	3.44	–	0.26	–
120	3.40	3.49	–	0.37	–
144	3.20	3.19	2.02	0.33	0.27
192	2.79	–	1.65		0.34

available experimental data do not provide sufficient information to identify its quantitative characteristics. The observed overall agreement between the model predictions and experimental data indicates that the postulated stochastic mechanism of oligodendrocyte generation *in vitro* is biologically plausible.

Table 2. Mean number of oligodendrocytes in the absence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_2(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
		–	0.27	–	0.08
48	0.18	1.05	–	0.12	–
72	1.00	2.57	–	0.16	–
96	2.38	4.26	–	0.24	–
120	3.95	5.21	2.69	0.25	0.33
144	5.50	–	4.37	–	0.36
192	8.33	–	–	–	–

Table 3. Mean number of O-2A progenitor cells in the presence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_1(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
		–	2.29	–	0.19
48	2.83	1.84	–	0.17	–
72	2.41	1.39	–	0.14	–
96	1.55	0.92	–	0.19	–
120	0.91	0.76	0.92	0.15	0.22
144	0.53	–	0.56	–	0.21
192	0.17	–	–	–	–

Table 4. Mean number of oligodendrocytes in the presence of thyroid hormone. Results from two independent experiments

Time (hours)	Expected value $M_2(t)$	Sample mean		Standard error	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
		–	0.54	–	0.10
48	0.68	2.09	–	0.13	–
72	2.41	3.73	–	0.19	–
96	4.06	5.75	–	0.34	–
120	5.16	5.81	3.68	0.28	0.29
144	5.81	–	5.42	–	0.37
192	6.40	–	–	–	–

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EVIDENCE APPENDIX
EXHIBIT 14

Bögler et al., "Measurement of Time in Oligodendrocyte-Type-2 Astrocyte (O-2A) Progenitors is a Cellular Process Distinct from Differentiation or Division," *Dev. Biol.* 162(2):525-38 (1994)

DEVELOPMENTAL BIOLOGY 162, 525–538 (1994)

Measurement of Time in Oligodendrocyte-Type-2 Astrocyte (O-2A) Progenitors Is a Cellular Process Distinct from Differentiation or Division

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When stimulated by platelet-derived growth factor (PDGF), oligodendrocyte-type-2 astrocyte (O-2A) progenitors derived from perinatal rat optic nerves undergo a limited number of cell divisions before clonally related cells synchronously and symmetrically differentiate into nondividing oligodendrocytes. The duration of this mitotic period is thought to be controlled by a cell-intrinsic biological clock. Thus, in the presence of PDGF, the measurement of time by the biological clock is intimately coupled to the control of division and differentiation. In contrast, O-2A progenitors grown in the presence of PDGF plus basic fibroblast growth factor (bFGF) divide indefinitely in the absence of differentiation and so do not exhibit a limited period of division. We have tested whether growth in PDGF plus bFGF alters the duration of the limited period of division O-2A progenitors exhibit in response to PDGF alone. Accordingly, O-2A progenitors were grown in the presence of PDGF plus bFGF for varying lengths of time, before being switched to conditions that promote timed differentiation (PDGF but not bFGF). Increasing duration of culture in PDGF plus bFGF led to a gradual shortening of the period for which O-2A progenitors were subsequently responsive to PDGF alone, until eventually all cells differentiated without dividing after switching. In contrast, a short exposure to bFGF was not sufficient to cause a similar alteration in the pattern of differentiation. These results indicate that O-2A progenitors prevented from undergoing timed differentiation nevertheless retain the ability to measure elapsed time, implying that the biological clock in this cell type can be uncoupled from differentiation. Furthermore, they demonstrate that the biological clock does not impose an absolute limit on the number of divisions that an O-2A progenitor can undergo. In contrast with existing hypotheses, our observations suggest that the molecular mechanism that controls timed differentiation must consist of at least two components, with the clock itself being in some manner distinct from mechanisms that limit cell division and/or directly regulate differentiation. © 1994 Academic Press, Inc.

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INTRODUCTION

Several observations in a variety of cell types suggest that measurement of elapsed time by cells is closely linked to the initiation of terminal differentiation. For example, hematopoietic stem cells generate erythroid cells which switch from production of fetal hemoglobin to adult hemoglobin after the passage of a seemingly preprogrammed length of time (Zanjani *et al.*, 1979; Wood *et al.*, 1985). Similarly, the number of divisions a fibroblast can undergo before terminally differentiating into a senescent cell appears to be preprogrammed or limited (reviewed in Goldstein, 1990). The measurement of elapsed time also seems to play a major controlling role in the timing of differentiation of glial precursor cells of the central nervous system (CNS)[2] into oligodendrocytes (Abney *et al.*, 1981; Temple and Raff, 1986; Noble *et al.*, 1988; Raff *et al.*, 1988; reviewed in Groves *et al.*, 1991, and Noble, 1991). The apparent coupling of the measurement of elapsed time to differentiation in these systems raises the question of whether these two processes are mechanistically distinct.

Understanding the regulatory mechanisms that make it possible for cells to differentiate according to an intrinsic schedule has been a subject of considerable inter-

[2] Abbreviations used: Ast, astrocyte oculture; Ast/PF, astrocyte coculture with additional exogenous PDGF; Ast/PF, astrocyte coculture with exogenous PDGF plus bFGF; bFGF, basic fibroblast growth factor; CNS, central nervous system; DMEM-BS, Dulbecco's Modified Eagle's Medium modified according to Bottenstein and Sato, 1979; DMEM-FCS, DMEM containing fetal calf serum; E19, Embryonic Day 19; ECM, extracellular matrix; O-2A, oligodendrocyte-type-2 astrocyte; P7, Postnatal Day 7; PDGF, platelet-derived growth factor; SV40 T, simian virus 40 large tumor antigen.

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est. Hypotheses about the nature of cellular timing mechanisms are as varied as the terms used to describe the phenomenon itself. Thus the terms "biological clock," "developmental clock," "time-measuring ability of cells," "finite mitotic life span," and other similar phrases have been used to describe phenomena in which a cell-intrinsic timing or measuring system appears to control the timing of differentiation. Due to the lack of knowledge about what is being measured by biological clocks, we describe this phenomenon as the measurement of elapsed time (see for example Orgel, 1973). This should be taken only as indicating that the passage of time is associated with the changes in the behavior of cells and is not meant to suggest that cells measure time in the same way that mechanical clocks do. It has been suggested, for example, that "biological time [is] equivalent to trains of specific physical or chemical events, which is a very different concept than that of an intrinsic clock based on sidereal or calendar time" (Finch, 1990). In the instances of particular interest here, it is clear that the duration of the period to be measured is determined at least several (and sometimes many) cell divisions before the differentiation event is itself observable. Most investigations of this phenomenon have been carried out on fibroblasts (which enter a nondividing senescent state after a limited number of cell divisions) and the range of hypotheses advanced to explain the limited mitotic life span of these cells includes random accumulation of cellular damage (Szilard, 1959; Orgel, 1973; Goldstein, 1990), telomere shortening (Harley *et al.*, 1990), changes in negative-growth regulatory genes (Weinberg, 1993), and progression through a genetic program (Orgel, 1973; Bayreuther *et al.*, 1988a,b; Goldstein, 1990). One could equally imagine that timed differentiation is caused by a steady increase, over a number of cell divisions, in the amount of a differentiation-inducing activity which determines cellular phenotype only after passing a threshold. Alternately, the steady decrease in the amount of some activity absolutely required for cell division could trigger the timed cessation of division.

All of the hypothetical mechanisms proposed to explain the workings of biological clocks appear to share the common feature of predicting that when the measuring process has been completed, differentiation (and, in at least some instances, cessation of division) follows necessarily. For example, if the period of division were limited by the accumulation of cellular damage or telomere shortening, then these events could not occur in cells stimulated to divide beyond their "normal" limit, for these events should by themselves be sufficient to limit cell division. Similarly, if the functioning of the biological clock relied simply on the buildup of a transcription factor to a level required to induce differentiation, then accumulation of such a factor should not occur in cells prevented from differentiating.

One experimental system that can be used to analyze the relationship among the measurement of elapsed time, the cessation of cell division, and the onset of differentiation is the timed differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells into oligodendrocytes. O-2A progenitors from embryonic rat CNS can be cultured in such a manner as to generate the first oligodendrocytes at a time equivalent to the day of birth, when the first oligodendrocytes appear *in vivo* (Abney *et al.*, 1981; Raff *et al.*, 1985, 1988). This appropriately scheduled differentiation in culture requires the presence of cortical astrocytes (Raff *et al.*, 1985) or purified platelet-derived growth factor (PDGF) (Raff *et al.*, 1988), an O-2A progenitor mitogen secreted by cortical astrocytes (Noble and Murray, 1984; Noble *et al.*, 1988; Richardson *et al.*, 1988). For example, in the presence of PDGF, optic nerve cultures from Embryonic Day 18 (E18) rats would generate the first oligodendrocytes after 3 days *in vitro*, while cultures from E19 rats would do so after 2 days (Raff *et al.*, 1988). That the scheduled differentiation of oligodendrocytes relied on the ability of O-2A progenitors to measure elapsed time was implied by the observation that clonally related O-2A progenitors generally ceased proliferating and differentiated within one division of each other, even if grown in separate tissue culture dishes after their first division (Temple and Raff, 1986). Synchronous differentiation of clonally related O-2A progenitors is also observed if cells are grown in chemically defined medium containing PDGF (Noble *et al.*, 1988; Raff *et al.*, 1988). Thus, existing observations suggest that the appropriately timed generation of oligodendrocytes relies on a cell-intrinsic clock that resides within the O-2A progenitor and measures cell divisions or some other parameter (Temple and Raff, 1986).

In contrast to the behavior of O-2A progenitors stimulated to divide with PDGF, the appropriately timed generation of oligodendrocytes does not occur if cultures of optic nerve cells are grown in the absence of mitogen or are treated simultaneously with PDGF and basic fibroblast growth factor (bFGF). In the absence of mitogen, O-2A progenitors differentiate rapidly and prematurely into oligodendrocytes without dividing (Raff *et al.*, 1983; Noble and Murray, 1984; Temple and Raff, 1985). The diametrically opposite result is obtained if O-2A progenitors are grown in the presence of PDGF plus bFGF, a condition in which continuous division of O-2A progenitors is maintained in the absence of differentiation (Bögler *et al.*, 1990; Groves *et al.*, 1993). These latter results indicate that O-2A progenitors are not intrinsically limited to a relatively small number of divisions

before differentiating. Rather, such a limit represents a pattern of division and differentiation observed only when cells are grown specifically in the presence of PDGF.

The ability to promote continuous division of O-2A progenitors in the absence of differentiation by treatment with PDGF plus bFGF has offered us the opportunity to study in more detail the relationship between the measurement of time by a biological clock and the onset of differentiation and cessation of division. Our data show that prolonged exposure to PDGF plus bFGF alters the behavior O-2A progenitors subsequently exhibit in response to PDGF alone. It is also shown that a brief exposure to bFGF, in the continued presence of PDGF, did not alter the timing of differentiation of O-2A progenitors into oligodendrocytes. These results suggest that the measurement of time still occurs under conditions in which division continues indefinitely in the absence of differentiation. This implies that the measurement of elapsed time can be separated mechanistically from the mechanisms that control the onset of differentiation or the cessation of cell division.

MATERIALS AND METHODS

Analysis of Small Populations and Clones of Optic Nerve Cells

Primary optic nerve cultures and purified cortical astrocytes were established as described previously (McCarthy and De Vellis, 1980; Raff *et al.*, 1983; Noble and Murray, 1984; Raff *et al.*, 1985). For the period of culture in the presence of PDGF plus bFGF optic nerve cells were seeded in poly-L-lysine-coated 25-cm^2 flasks at 200,000 to 300,000 cells per flask in DMEM-BS, a chemically defined medium (a modification of the medium described by Bottenstein and Sato, 1979; Bögler *et al.*, 1990). Bulk optic-nerve-cell cultures were given 10 ng/ml of recombinant human PDGF A-chain homodimer [Chiron Corporation (a kind gift of Dr. C. George-Nascimento) or Promega] and recombinant human bFGF (Boehringer-Mannheim or Promega) each day and half the medium was changed every other day. Once a week the cells were passaged by trypsinization in calcium- and magnesium-free DMEM containing 200 μg/ml EDTA and 6000 U/ml trypsin, followed by trypsin inhibitor (added 1 part in 3.5; 0.52 mg/ml soybean trypsin inhibitor, 0.04 mg/ml bovine pancreas DNase, and 3 mg/ml BSA fraction V in DMEM; Sigma). An aliquot of cells was removed for analysis at the times indicated under the Results section. The remainder of the cells were returned to bulk culture as above.

For analysis of small populations of optic nerve cells (Fig. 1), cocultures were established with cortical astrocytes, which produce PDGF (Noble *et al.*, 1988) and maximize optic nerve cell viability (Temple and Raff, 1986; Raff *et al.*, 1988; see also Barres et al., 1992). As the age of the cortical astrocyte cultures could conceivably affect the behavior of O-2A progenitors (see Lillien and Raff, 1990, for one such phenomenon), fresh cultures of astrocytes were prepared according to identical schedules for each analysis. Astrocyte cultures were equivalent to P21 at the time that optic nerve cells were plated on them, an age when O-2A progenitor division and differentiation are still occurring in vivo (Miller *et al.*, 1985). Furthermore, these astrocyte cultures were made in the same way as those used previously to establish that astrocytes make PDGF (Noble *et al.*, 1988; Raff *et al.*, 1988). Monolayers of 20,000 cortical astrocytes per poly-L-lysine-coated coverslip (Chance Propper No. 1, 13 mm) were cultured in 0.5 ml of DMEM containing 10% fetal calf serum (DMEM-FCS; Gibco BRL), which was changed 2 days later. After 3 days the cultures were irradiated with 2000 rads of X rays, washed once, and then fed with 0.5 ml DMEM-BS. One day later 100 μl of filter-sterilized DMEM-BS conditioned for 48 hr by cortical astrocytes (from a flask of the same cortex preparation that had not been passaged) was added to each well shortly before the optic nerve cells. Five hundred optic nerve-derived cells were delivered to the supernatant of these cultures in 10 μl. Approximately 50 to 100 cells were observed after 1 day on the coverslips covering part of the bottom of the well (Fig. 1). Half of the medium was replaced every other day, and growth factors were added daily.

For analysis of O-2A progenitor clones (Fig. 2) 1000 cortical astrocytes were plated in 10 μl of DMEM-FCS per Terasaki microwell and the plates incubated upside down for the first 48 hr, so that the astrocytes attached predominantly to the sides of the wells. This facilitated observation of the optic nerve cells subsequently plated into the microwells. These cultures were treated identically to those on coverslips described above, except: (a) instead of changing the medium after 2 days, 10 μl of DMEM-FCS was added; (b) they were not washed after irradiation before the medium was replaced with 10 μl of DMEM-BS; and (c) no conditioned medium was added separately, but the final dilutions of optic nerve cells were made in astrocyte-conditioned medium. A variety of dilutions of optic nerve-derived cells, designed to deliver between 1 and 10 cells per 10 μl per well, were plated into the Terasaki wells. The next day, and every 2 days after that, 10 μl of the medium was removed and the cultures were observed, and 10 μl DMEM-BS containing 10 μg/ml PDGF was replaced. After oligodendrocytic differentiation was judged to be complete on the basis of the morphology of visible cells, the cells

TABLE 1
bFGF ATTACHES TO THE MATRIX OF E19 OPTIC NERVE CULTURES

	E19 cells grown for first 24 hr in:	ECM incubated with:	Indicator cells cultured in:	% O-2A lineage cells GalC+ after 4 days in vitro
1	—	—	PDGF	46.9 ± 3.2
2	—	—	PDGF + bFGF	3.3 ± 0.4
3	PDGF	PDGF	PDGF	57.4 ± 2.3
4	PDGF	PDGF	PDGF + bFGF	5.1 ± 0.8
5	bFGF	—	PDGF	4.0 ± 0.4
6	bFGF	—	PDGF + bFGF	3.2 ± 0.9
7	—	bFGF	PDGF	3.7 ± 0.4
8	—	bFGF	PDGF + bFGF	4.3 ± 0.6

Note. Exposure of E19 optic nerve cultures to bFGF either before or after lysis results in an extracellular matrix that inhibits the differentiation of O-2A progenitors in conjunction with PDGF. E19 optic nerve cultures were grown in the absence of growth factor (rows 1, 2, 7, and 8) in the presence of PDGF (rows 3 and 4) or in the presence of bFGF (rows 5 and 6) before they were lysed by hypotonic shock. The ECM was then incubated for 24 hr in the absence of growth factor (rows 1, 2, 5, and 6) in PDGF (rows 3 and 4) or bFGF (rows 7 and 8) before indicator cells derived from 7-day-old optic nerve were plated onto the ECM and grown for a further 4 days in the presence of PDGF (odd-numbered rows) or PDGF plus bFGF (even-numbered rows). Data are presented as means ± SEM of three experiments.

were prepared for immunocytochemistry. The time required for oligodendrocytic differentiation to appear complete varied from culture to culture and depended on the size of the clones. The smallest clones (Fig. 2C) appeared to have differentiated after 3 days or less and were prepared for immunocytochemistry at that time. The largest clones were prepared for immunocytochemistry 10 days after optic nerve cells were plated into the microwells. Observations made before and after immunocytochemistry were compared, and only if they were sufficiently similar so that it appeared beyond reasonable doubt that a group of cells was derived from the single O-2A progenitor observed on Day 1 was the clone allowed into the data set shown in Fig. 2.

Analysis of Embryonic Optic Nerve Cultures

Initial experiments showed that E19 cultures that were given bFGF only on Day 1 behaved identically to those that received bFGF every day over a 10-day period: no oligodendrocytes appeared (data not shown; all cultures received PDGF every day). One possibility was that bFGF was persisting in these cultures due to interactions with the extracellular matrix (ECM; for review see Gospodarowicz *et al.*, 1987; see also Lillien and Raff, 1990). To investigate this directly, the interaction of bFGF with the ECM of E19 optic nerve cultures was studied. E19 optic nerve cells were plated on coverslips and cultured in the absence of factors or in the presence of either PDGF or bFGF (10 ng/ml each) for 24 hr. These cultures were then subjected to hyposmotic lysis after 24 hr to generate an adherent cell lysate which consists predominantly of ECM (as in Lillien and Raff, 1990). Coverslips were then washed once with PBS and twice with DMEM-BS, incubated for 24 hr in DMEM-BS, or in DMEM-BS containing either PDGF or bFGF (20 ng/ml), and washed twice in DMEM-BS. Indicator cells from P7 optic nerve cultures maintained in PDGF plus bFGF for 2 days were then plated onto the ECM and grown for a further 4 days in the presence of PDGF or PDGF plus bFGF (10 ng/ml each) before they were prepared for immunocytochemistry. If bFGF was present either before lysis, or after lysis but before the indicator cells were added, differentiation was inhibited as much as if bFGF was present after the indicator cells were added (Table 1, all indicator cell cultures received PDGF). In contrast, if the cultures saw either no growth factors before the indicator cells were plated, and then only PDGF, or if they saw only PDGF throughout, considerable differentiation occurred (Table 1). Therefore, it appears that bFGF became strongly attached to the ECM of these cultures and was able to affect the differentiation of O-2A progenitors in that attached form. In order to be sure to eliminate any bFGF attached to the ECM after the first 24 hr of culture, E19 optic nerve cultures were established in bulk (as above) and passaged by trypsinization after 24 hr. Cells were then plated on poly-L-lysine-coated coverslips at a density of 10,000 cells/coverslip in DMEM-BS. Cultures received either PDGF or PDGF plus bFGF throughout, or PDGF plus bFGF for the first 24 hr and then PDGF on a daily basis, at 10 ng/ml each. Approximately half the DMEM-BS was changed every 2 days, and cells were prepared for immunocytochemistry at the times indicated.

PDGF Receptor Analysis

For the PDGF receptor analysis shown in Figs. 4 and 5, optic nerve cultures were established in bulk and treated as described earlier. Aliquots of cells removed for analysis were plated directly onto poly-L-lysine-coated coverslips at a density of 3000 cells per coverslip. They were cultured for 48 hr in the presence of PDGF or PDGF plus bFGF (10 ng/ml each) and prepared for immunocytochemistry.

Immunocytochemistry

The antibodies used were monoclonal anti-galactocerebroside (anti-GalC) antibody (Ranscht *et al.*, 1982) and monoclonal antibody A2B5 (Eisenbarth *et al.*, 1979).

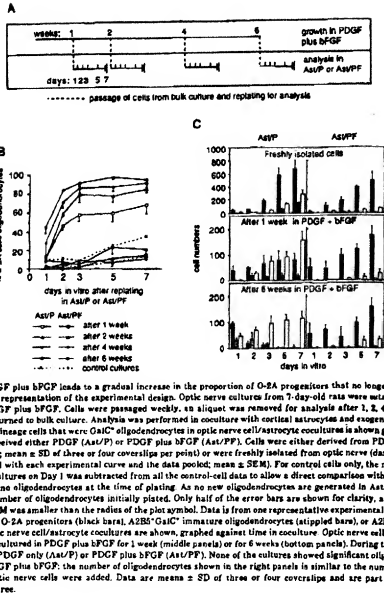

FIG. 1. Culture in PDGF plus bFGF leads to a gradual increase in the proportion of O-2A progenitors that no longer divide in response to AstP. (A) A schematic representation of the experimental design. Optic nerve cultures from 7-day-old rats were established in bulk for the period of culture in PDGF plus bFGF. Cells were passaged weekly, an aliquot was removed for analysis after 1, 2, 4, and 6 weeks, and the remaining cells were returned to bulk culture. Analysis was performed in coculture with cortical astrocytes and exogenous PDGF (AstP). (B) The percentage of O-2A lineage cells that were GalC$^+$ oligodendrocytes in optic nerve cell/astrocyte cocultures is shown graphed against time in coculture. Cocultures received either PDGF (AstP) or PDGF plus bFGF (AstPF). Cells were either derived from PDGF plus bFGF-treated bulk cultures (solid lines; mean ± SD of three or four coverslips per point) or were freshly isolated from optic nerve (dashed lines; control cells were analysed in parallel with each experimental curve and the data pooled; mean ± SEM). For control cells only, the number of oligodendrocytes found in AstP/PF cultures on Day 1 was subtracted from all the control-cell data to allow a direct comparison with experimental cultures which contained almost no oligodendrocytes at the time of plating. As no new oligodendrocytes are generated in AstP/PF this number most closely resembles the number of oligodendrocytes initially plated. Only half of the error bars are shown for clarity, and if no error bars are shown then the SD or SEM was smaller than the radius of the plot symbol. Data is from one representative experimental series of three. (C) The numbers of A2B5$^+$GalC$^+$ O-2A progenitors (black bars), A2B5$^+$GalC$^+$ immature oligodendrocytes (stippled bars), or A2B5$^+$GalC$^+$ oligodendrocytes (white bars) in optic nerve cell/astrocyte cocultures are shown, graphed against time in coculture. Optic nerve cells were freshly isolated (top panels) or had been cultured in PDGF plus bFGF for 1 week (middle panels) or for 6 weeks (bottom panels). During the time shown parallel cultures received either PDGF only (AstP) or PDGF plus bFGF (AstPF). None of the cultures showed significant oligodendrocytic differentiation if cultured in PDGF plus bFGF; the number of oligodendrocytes shown in the right panels is similar to the numbers seen in astrocyte cultures to which no optic nerve cells were added. Data are means ± SD of three or four coverslips and are part of one representative experimental series of three.

The A2B5 monoclonal antibody specifically labels O-2A lineage cells in these cultures and is an IgM (Raff *et al.*, 1983), while anti-GalC, an IgG3, specifically labels oligodendrocytes (Raff *et al.*, 1978). Fluorescein- and rhodamine-conjugated second-layer antibodies against monoclonal antibodies were from Southern Biotechnology and were used at a dilution of 1:100. Fluorescein-conjugated anti-rabbit antibodies were from Tioga (USA) and were used at 1:200. Standard immunofluorescence to identify cells of the O-2A lineage was performed as described (Raff *et al.*, 1983; Noble and Murray, 1984; Bögler *et al.*, 1990). Anti-PDGF receptor immunofluorescence was done as follows. Cultures were rinsed in phosphate-buffered saline, fixed for 10 min in 4%

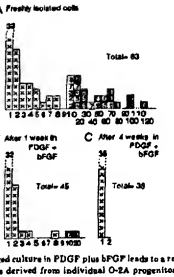

FIG. 2. Prolonged culture in PDGF plus bFGF leads to a reduction in the size of clones derived from individual O-2A progenitors. A stem and leaf plot (as in Temple and Raff, 1986) showing the reduction in clone size in the presence of astrocytes and PDGF, after switching from culture in PDGF plus bFGF. Each 's' or number represents one clone. To obtain the number of cells in a given clone either read the number below the column in which the 's' is or add the number in the graph (units) to the number under the column (tens). For example in the upper panel there are three clones of five cells and five clones that are between 10 and 20 cells consisting of 12, 12, 13, 17, and 18 cells. The total number of clones analyzed under each condition is indicated. The experimental results are from two separate bulk cultures that were analyzed after 1 and 4 weeks of culture in PDGF plus bFGF; data from freshly isolated P7 rat optic nerve cultures analyzed in parallel with each experiment were pooled. Clones shown in black writing on white ground consisted entirely of GalC$^+$ oligodendrocytes; clones shown as white writing on black ground contained some A2B5$^+$ GalC$^-$ O-2A progenitors, and these cells always comprised less than a third of the total cells, and typically about 10%.

paraformaldehyde, and then exposed sequentially to the following in Hepes-buffered Hanks' balanced salt solution (Imperial Laboratories) containing 5% newborn calf serum and 5% goat serum (both from GibcoBRL): 25 min A2B5 and anti-GalC, 25 min anti-IgG-biotin, 25 min streptavidin-coumarin (Molecular Probes, Oregon) and anti-IgM-fluorescein, 10 min 0.1% v/v Triton X-100, 45 min anti-PDGF receptor antibody (1:500; UBI), two 5-min washes, 30 min anti-rabbit fluorescein, two 5-min washes. Cultures were viewed on a Zeiss Axiphot microscope equipped with phase contrast and epi-uv illumination and selective filters for fluorescein, rhodamine, and coumarin.

Immunoprecipitation and Western Blot Analysis

Cultures of NIH 3T3 mouse fibroblasts, Rat-2 fibroblasts, and O-2A progenitors were harvested before becoming confluent by rinsing in PBS and scraping in 600 μl Ripa buffer (150 m*M* NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 m*M* Tris, pH 8.0) containing 0.5 m*M* PMSF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 40 μg/ml bestatin, and 1 μg/ml aprotinin. Insoluble material was removed by centrifugation, and the supernatant was immunoprecipitated by the addition of 20 μl of R7 anti-PDGF receptor antiserum (Eriksson *et al.*, 1992) and 60 μl of protein A-Sepharose CL4B (Pharmacia) and incubating on ice for 60 min. The immunoprecipitate was washed three times in 500 μl of Ripa buffer, and an equal volume of 2x sample buffer was added. The precipitates were boiled, separated on an acrylamide gel, and blotted to Immobilon P membrane (described in Harlowe and Lane, 1988). The immunoblot was preblocked for 4 hr in blotto (5% nonfat dry milk in phosphate-buffered saline, 0.02% azide), exposed overnight to R7 antibody (1:400), washed extensively and exposed to ^{125}I-donkey anti-rabbit antibodies for 1 hr, followed by several washes, and exposed to X ray film.

RESULTS

O-2A progenitors dividing and differentiating in the presence of cortical astrocytes or PDGF exhibit three observable behaviors from which the functioning of their cell-intrinsic clock can be inferred. The first behavior is the ability of populations of O-2A progenitors to continue to generate new oligodendrocytes for a prolonged period of time, a process that is dependent upon ongoing O-2A progenitor division and differentiation (Noble and Murray, 1984; Temple and Raff, 1986; Noble *et al.*, 1988; Raff *et al.*, 1988). In contrast, if O-2A progenitors are no longer able to divide, oligodendrocyte numbers rapidly increase initially and then show no further change when all the O-2A progenitors have differentiated (assuming that the fairly consistent rate of differentiation of individual cells (e.g., Raff *et al.*, 1984) is itself not changed by the experimental manipulations). The second behavior that can be observed is the generation of clones of oligodendrocytes derived from a single O-2A progenitor. The size of the clone depends on the length of the limited period of division elicited by PDGF and so reflects the period of time that remained to be measured by the founding O-2A progenitor before differentiation was initiated (Temple and Raff, 1986). The third behavior is exhibited by populations of O-2A progenitors derived from embryonic optic nerve. Such populations give rise to the first oligodendrocytes *in vitro* at a time equivalent to the day of birth (Noble *et al.*, 1988; Raff *et al.*, 1988). This last behavior is the measure most likely to indicate whether the biological clock in O-2A progenitors is functioning appropriately.

The effect of preventing differentiation while stimulating division for varying periods of time has been experimentally determined for all three of these behaviors. The experimental design used for the analyses of the first two behaviors (experiments reported in Figs. 1 and 2) was to grow O-2A progenitors (derived from the optic nerves of 7-day-old rats) under conditions that prevented differentiation (PDGF plus bFGF) and to switch cells after different durations of culture to conditions that promoted timed differentiation (PDGF but not bFGF). The ability of these cells to divide and differentiate was then compared to that of freshly isolated control O-2A progenitors. Accordingly, optic nerve cells were established in bulk culture and given PDGF plus bFGF daily. Cultures were passaged weekly, and aliquots of cells were removed and replated in the presence of purified cortical astrocytes for analysis (see Fig. 1A). Coculture with cortical astrocytes was used as astrocytes make PDGF and promote timed differentiation of O-2A progenitors (Noble *et al.*, 1988; Richardson *et al.*, 1988) as well as maximizing O-2A lineage cell viability (Temple and Raff, 1986; Barres *et al.*, 1992), thereby allowing the analysis of small populations of cells or even individual clones. Astrocyte-optic nerve cocultures received either no additional factors, PDGF, or PDGF plus bFGF (referred to as Ast, Ast/P, or Ast/PF, respectively) and were analyzed by immunocytochemistry after 1, 2, 3, 5, and 7 days. As expected from the fact that astrocytes make PDGF, we observed no differences between cultures that received no additional factors (data not shown) and those that received PDGF alone and present data only on cells that were switched to Ast/P.

Populations of freshly isolated O-2A progenitors cultured in the presence of PDGF exhibit both O-2A progenitor self-renewal by division and oligodendrocyte generation by differentiation (Noble and Murray, 1984; Noble *et al.*, 1988; see also Wren *et al.*, 1992). In PDGF-treated cultures of optic nerve cells, the extent of O-2A progenitor self-renewal is related to the proportion of O-2A progenitors with some time remaining in their limited period of division, and so have not undergone timed differentiation. The capacity of O-2A progenitor self renewal to continue for prolonged periods in populations of cells is thought to reflect a heterogeneity in the duration of the mitotic period remaining in different clones of the starting population (Temple and Raff, 1986). As a consequence one cannot measure the timed differentiation of O-2A progenitors into oligodendrocytes in postnatal optic nerve cultures as a single endpoint. The changing numbers of O-2A progenitors and oligodendrocytes in a population analyzed at various time points can be used, however, to give some indication of the amount of time that remains to be measured by the

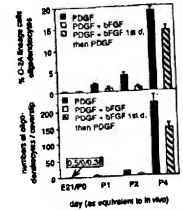

FIG. 2. Transient exposure to PDGF plus bFGF does not perturb the appearance of the first oligodendrocytes in embryonic optic nerve cultures. Data shows the percentage of O-2A lineage cells that were GalC$^+$ oligodendrocytes (top) or the average number of oligodendrocytes seen per coverslip (bottom) in cultures derived from E19 optic nerve. The average number of oligodendrocytes per coverslip at the time equivalent to E21/P0 is shown as numbers (PDGF:0.5/PDGF plus bFGF:0/PDGF plus bFGF for first day, then PDGF, 0.36), as the bars are too small to be seen. Cultures were established in bulk for the first 24 hr and then passaged and replated on coverslips. They received either PDGF throughout, PDGF plus bFGF throughout, or PDGF plus bFGF for the first 24 hr and then PDGF only. Differentiation state was analysed by immunocytochemistry at 2, 3, 4, and 6 days after dissection, shown as days equivalent to in vivo. Data are presented as means ± SEM of three experiments.

O-2A progenitors in that population. If, on average, a large amount of time remains in the limited period of division that precedes timed differentiation, then one would observe the continuous generation of both O-2A progenitors and oligodendrocytes. In contrast, if all the O-2A progenitors have completed the measurement of time and reached the end of their period of division, they would be expected to differentiate rapidly into oligodendrocytes and to show little sign of self-renewal.

Culture of O-2A Progenitors in PDGF Plus bFGF Leads to a Gradual Reduction in the Duration for Which They are Subsequently Mitotically Responsive to PDGF Alone

Analysis of populations of O-2A progenitors that had been switched from culture in PDGF plus bFGF to Ast/P showed a dramatic increase in the proportion of O-2A lineage cells that were oligodendrocytes after the first 3 days in Ast/P, when compared to freshly isolated O-2A progenitors (Fig. 1B). Furthermore, the proportion of the starting population that differentiated into oligodendrocytes during the first 3 days of culture in Ast/P

TABLE 2
O-2A PROGENITORS THAT ARE NO LONGER MITOTICALLY RESPONSIVE TO PDGF EXPRESS PDGF RECEPTORS

Time of culture in PDGF plus bFGF	Percentage of O-2A progenitors that were PDGF receptor$^+$	
	PDGF	PDGF + bFGF
Control cells	96.1 ± 1.1	95.9 ± 1.1
1 week	96.0 ± 1.4	96.8 ± 0.9
2 weeks	98.1 ± 0.5	98.0 ± 0.7
4 weeks	99.6 ± 0.3	98.7 ± 0.5
6 weeks	97.9 ± 0.8	98.3 ± 0.5

Note. The percentage of O-2A progenitors that were PDGF receptor$^+$ after various times of culture in the presence of PDGF plus bFGF followed by a further 2 days of culture in PDGF or PDGF plus bFGF. For culture in the presence of PDGF plus bFGF cells were treated as described for the experiments shown in Figs. 1 and 2. Aliquots of cells were removed after 1, 2, 4, and 6 weeks and replated at a density of 3000 cells per coverslip. They were treated with either PDGF or PDGF plus bFGF and prepared for immunocytochemistry after 2 days. They were labeled with anti-PDGF receptor antibodies, as well as A2B5 and anti-GalC antibodies. The proportion of A2B5$^-$ GalC$^-$ O-2A progenitors that were also labeled by the anti-PDGF receptor antibodies is shown. Data is mean ± SEM for between 6 and 10 coverslips from two separate experiments.

correlated well with the duration of prior culture in PDGF plus bFGF (Fig. 1B): the longer cells had been grown in the presence of PDGF plus bFGF the greater the proportion of O-2A lineage cells that differentiated into oligodendrocytes after 3 days in Ast/P. Three days after switching cells to Ast/P the rate of increase of the percentage of oligodendrocytes slowed, either because the rate of O-2A progenitor self-renewal became similar to the rate of oligodendrocyte generation (after 1, 2, and 4 weeks in PDGF plus bFGF) or because there were almost no O-2A progenitors left at this time (after 6 weeks in PDGF plus bFGF). As expected, all cultures in Ast/PF showed little increase in the proportion of O-2A lineage cells that were oligodendrocytes as under these conditions O-2A progenitor division occurred in the absence of oligodendrocyte differentiation (Fig. 1B).

Examination of the data shown in Fig. 1B in terms of cell numbers suggested that after 6 weeks in PDGF plus bFGF, no significant amount of O-2A progenitor division occurred in Ast/P (Fig. 1C, bottom left panel shows no change in the sum of the bar heights; the culture conditions prevent any significant cell death: see Barres *et al.*, 1992). In contrast, freshly isolated populations of O-2A progenitors, or cells switched after 1 week of culture in PDGF plus bFGF, showed a combination of O-2A progenitor self-renewal and oligodendrocyte generation in Ast/P (Fig. 1C, left panels). The behavior of O-2A progenitors switched to Ast/P after only 1 week of culture in PDGF plus bFGF was intermediate between that of the cells grown for 6 weeks and freshly isolated cells: although some O-2A progenitor division occurred (as indicated by an increase in cell number), a larger proportion of cells in the 1 week pre-treated culture became oligodendrocytes within 3 days than in cultures of freshly isolated cells (compare Fig. 1C left panels). As expected, all Ast/PF cultures showed O-2A progenitor division and no significant oligodendrocyte generation over the time points examined (Fig. 1C).

Cells differentiating from an A2B5$^+$GalC$^-$ O-2A progenitor into an A2B5$^-$GalC$^-$ oligodendrocyte are transiently A2B5$^+$GalC$^+$, and the proportion of O-2A lineage cells that occupy this compartment at any given time is related to the rate of differentiation of the population as a whole. Examination of this A2B5$^+$GalC$^+$ compartment also shows that pretreatment with PDGF plus bFGF is associated with an increase in the number of differentiating cells seen at early time points (Fig. 1C, stippled bars). Again, 6 weeks of prior culture in PDGF plus bFGF had a more marked effect than 1 week of prior culture (Fig. 1C, left panels). After 6 weeks in the presence of PDGF plus bFGF, very few A2B5$^+$GalC$^-$ cells were found in these culture after the 3-day time point (Fig. 1C). In contrast, A2B5$^+$GalC$^+$ cells were found on all days examined in cultures grown in the presence of PDGF plus bFGF for 1 week before switching to Ast/P. It is interesting to note, however, that significant numbers of such cells were seen in these cultures several days before their presence in cultures derived from freshly isolated cells. Thus, these data also indicate that the yield of oligodendrocytes within a given time period of growth in Ast/P is increased by prior culture in PDGF plus bFGF and further indicate that the rate of differentiation itself is probably not altered from the figure of 3 days reported in earlier studies (Raff *et al.*, 1984).

Culture of O-2A Progenitors in PDGF Plus bFGF Leads to a Stepwise Reduction in the Size of the Oligodendrocyte Clones Subsequently Generated in Response to PDGF Alone

Clonal analysis allows direct measurement of the number of cell divisions that the founding cell of a clone and its progeny underwent. In the case of O-2A progenitors dividing in response to PDGF, clone size depends on the length of the limited mitotic period that precedes timed differentiation, assuming that no significant amount of cell death occurred (Temple and Raff, 1986). Therefore, clones derived from O-2A progenitors that

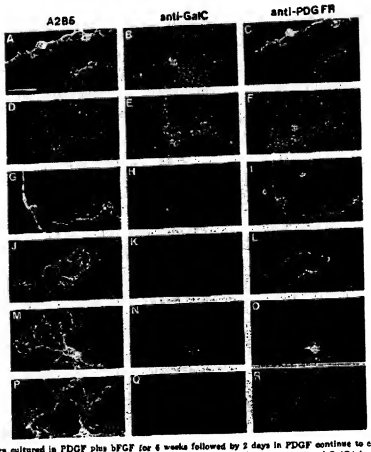

FIG. 4. O-2A progenitors cultured in PDGF plus bFGF for 6 weeks followed by 2 days in PDGF continue to express PDGF receptors. Three-color immunocytochemistry of optic nerve cultures using monoclonal A2B5 (via rhodamine) and anti-GalC (via coumarin) and polyclonal anti-PDGF receptor antibodies (via fluorescein). Cells were either derived directly from P7 optic nerve (A to I) or had undergone 6 weeks of preculture in PDGF plus bFGF (J to R). They were cultured for 2 days in the presence of PDGF alone (A to F and J to O) or in the presence of PDGF plus bFGF (G to I and P to R). A2B5$^+$GalC$^-$ O-2A progenitors were PDGF receptor$^+$ under all conditions (A to C, G to L; P to R). In contrast, A2B5$^+$GalC$^+$ oligodendrocytes such as the one indicated by the arrow in B, were negative for the PDGF receptor. D to F and M to O show A2B5$^+$GalC$^+$ immature oligodendrocytes that are PDGF receptor$^+$. In the control cultures that were derived from P7, optic nerve cells that were PDGF receptor$^+$, but did not label with either A2B5 or anti-GalC, were common (short arrows in I). These cells were probably pial meningeal cells. The scale bar in A is 30 μm.

had been grown in PDGF plus bFGF for varying periods of time, or had been freshly isolated from optic nerve, were analyzed in insulin containing coculture with cortical astrocytes (Noble *et al.*, 1988; Raff *et al.*, 1988; Barres *et al.*, 1992), following the method of Temple and Raff (1986). Cultures were observed the day after optic nerve cells were placed in microculture and every other day after that. When oligodendrocytic differentiation was judged to be complete by cell morphology (after between 3 and 10 days, depending on how many rounds of division occurred) cultures were prepared for immunocytochemistry to confirm the differentiation state of the cells and to facilitate counting of the cells.

Clones derived from freshly isolated O-2A progenitors showed a wide range of sizes as expected (Fig. 2A; Temple and Raff, 1986). In contrast, after 4 weeks in PDGF plus bFGF all of the 38 O-2A progenitors analyzed generated clones of 1 oligodendrocyte (Fig. 2C), suggesting that these O-2A progenitors were no longer induced to divide by PDGF when grown in single-cell microculture. None of the founding cells was seen to divide and all differentiated within 3 days of plating

FIG. 3. O-2A progenitors cultured for 7 weeks in PDGF plus bFGF express PDGF-α receptor. Anti-PDGF-α receptor Western blot of anti-PDGF-α receptor immunoprecipitates of Rat-2, NIH 3T3 fibroblasts, and an optic nerve culture that had been grown for 7 weeks in the presence of PDGF plus bFGF. The position of the PDGF-α receptor is indicated, and these bands migrated at approximately 180 kDa.

into the microwells. O-2A progenitors that had been first grown for a week in PDGF plus bFGF showed behavior that was intermediate between that of freshly isolated cells and cells that had been in the presence of PDGF plus bFGF for 4 weeks (Fig. 2B). The largest clone derived from freshly isolated cells consisted of 120 cells, implying that the founder O-2A progenitor of this clone underwent at least seven divisions. After 1 week in PDGF plus bFGF clones of 21 cells or fewer were obtained, suggesting that O-2A progenitors underwent a maximum of five divisions after switching to Ast/P. It should be noted that some of the clones shown in Fig. 2 contained A2B5$^+$GalC$^-$ O-2A progenitors at the time that the cultures were fixed (white letters on black background). These cells had appeared to be oligodendrocytes when they were observed live under the phase-contrast microscope as they had a more complex, branched morphology than typical bipolar O-2A progenitors. As in the course of oligodendrocytic differentiation, morphology changes before antigenic phenotype (Noble and Murray, 1984; Raff *et al.*, 1985; Temple and Raff, 1986) and is generally associated with a cessation of division (Small *et al.*, 1987; Noble *et al.*, 1988), it is most likely that these cells were at an early stage of differentiation. The presence of a few A2B5$^+$GalC$^-$ multipolar O-2A progenitors in some clones is consistent with previous observations (Temple and Raff, 1986).

A Short Exposure to bFGF Does Not Alter the Timing of the Appearance of the First Oligodendrocytes in PDGF-Treated Embryonic Optic Nerve Cultures

Analysis of the behavior of small populations (Fig. 1) and clones (Fig. 2) suggested that culture in PDGF plus bFGF led to a gradual shortening of the period of time during which O-2A progenitors would subsequently divide in response to Ast/P. One possible explanation is that cells registered the passing of time in the presence of PDGF plus bFGF and that exposure to bFGF in the presence of PDGF did not prevent the measurement of time in cultures of postnatal optic nerve. An alternative explanation would be that any exposure to bFGF dramatically increases the yield of oligodendrocytes produced within the first few days of subsequent growth in Ast/P. To test this possibility we used the sensitive assay for the correct functioning of the clock provided by the timed appearance of oligodendrocytes in cultures of embryonic optic nerve. The first oligodendrocytes appear in embryonic optic nerve cultures treated with PDGF at a time equivalent to birth, and invariably do so within a 24-hr window (Raff *et al.*, 1988). Therefore, we exposed embryonic optic nerve cultures transiently to bFGF (in the continued presence of PDGF) and followed the appearance of oligodendrocytes.

Transient exposure to bFGF in the continued presence of PDGF did not alter the time of appearance of the first oligodendrocytes in embryonic optic nerve cultures from that observed in control cultures that received PDGF throughout the experiment (Fig. 3). However, slightly fewer oligodendrocytes appeared over the course of the experiment in cultures that transiently received bFGF, both in terms of cell numbers and proportion of O-2A lineage cells. This raises the possibility that short-term exposure to bFGF increased the number of divisions that some clones of cells underwent, as has been suggested by others (McKinnon *et al.*, 1990). Cells that received either PDGF or PDGF plus bFGF throughout behaved as expected from previous studies: in PDGF alone the first oligodendrocytes were generated after 2 days (Fig. 3; Raff *et al.*, 1988), while in PDGF plus bFGF no oligodendrocytes were generated (Fig. 3; Bögler *et al.*, 1990).

O-2A Progenitors That Are Mitotically Unresponsive to PDGF Continue to Express PDGF-α Receptors

One possible explanation for the observation that prolonged culture in the presence of PDGF plus bFGF led to an inability of some or all O-2A progenitors to subsequently respond to Ast/P is that the levels of PDGF-α receptor declined. In order to investigate this possibility, we asked whether long-term cultures of O-2A progenitors grown in the presence of PDGF and bFGF continued to express PDGF-α receptors. We also tested whether O-2A progenitors grown for various periods of time in PDGF plus bFGF and then switched to the presence of PDGF alone for 2 days expressed PDGF-α receptors. Bulk optic nerve cultures derived from P7 optic nerve were established as for previous experiments. Cells were removed from PDGF plus bFGF after 1, 2, 4, and 6 weeks and analyzed by PDGF receptor immunocytochemistry after a further 2 days, either in

the presence of PDGF plus bFGF or in PDGF alone (Table 2 and Fig. 4). This time point was chosen as it represents a compromise between allowing the maximum time for the levels of receptor to decline if it were no longer being synthesized and retaining enough A2B5$^+$GalC$^-$ O-2A progenitors present in all the cultures to be able to analyze a reasonably sized population of cells.

Greater than 95% of A2B5$^+$GalC$^-$ O-2A progenitors showed a clear reaction with anti-PDGF receptor antibodies in all cultures examined, suggesting that they expressed PDGF-α receptor (Table 2 and Fig. 4) despite being refractory to the mitogenic effects of PDGF. In contrast, A2B5$^-$ GalC$^+$ oligodendrocytes were not labeled by the anti-PDGF receptor antibodies in any cultures (Fig. 4A to 4C). In both the control and experimental populations that received only PDGF, A2B5$^+$ GalC$^-$ immature oligodendrocytes that were also PDGF receptor$^+$ were seen, suggesting that at least in some cases PDGF-α receptor levels did not decline until after differentiation was already in progress.

The cells that remained from the bulk culture of one of the above experiments after 6 weeks were returned to culture in the presence of PDGF plus bFGF for a further 7 days before being harvested for analysis by immunoprecipitation and Western blotting with an antiserum specific for the PDGF-α receptor (Eriksson *et al.*, 1992). As can be seen in Fig. 5 this optic nerve culture, which contained almost only O-2A progenitors, expressed levels of PDGF-α receptor similar to NIH 3T3 or Rat-2 fibroblasts.

DISCUSSION

When grown in the presence of PDGF, O-2A progenitors derived from optic nerves of perinatal rats undergo a limited period of division, at the end of which they switch from division to differentiation. The duration of this period of division is thought to be regulated by a cell-intrinsic clock. We examined whether O-2A progenitor behavior changed with elapsed time when cells were grown for varying periods of time under conditions that stimulate division and prevent differentiation. It was found that O-2A progenitors grown in the presence of PDGF plus bFGF, which together prevent differentiation, gradually became refractory to the mitogenic effect of subsequent culture in Ast/P in the absence of bFGF. The longer the period in PDGF plus bFGF, the greater the proportion of O-2A progenitors that differentiated without dividing after having been switched to Ast/P. Eventually, after 6 weeks in PDGF plus bFGF, almost all the O-2A progenitors differentiated without dividing in Ast/P. In addition, O-2A progenitors precultured in PDGF plus bFGF gave rise to smaller clones of oligodendrocytes, after switching to single cell culture in Ast/P, than did freshly isolated cells grown in parallel single cell microculture. After 1 week in PDGF plus bFGF the maximum clone size observed after switching to Ast/P was 21 cells compared to 120 cells seen in the largest clone derived from freshly isolated O-2A progenitors. Exposure to both mitogens for 4 weeks was sufficient to produce a population of O-2A progenitors that no longer divided after switching to Ast/P in single cell microculture. In contrast, a short exposure to PDGF plus bFGF did not result in any similar shortening of the period of division of O-2A progenitors after switching to PDGF alone.

We suggest that the simplest explanation of our results is that O-2A progenitors cultured in the presence of PDGF plus bFGF measure time and retain a memory of the fact that they have exceeded the number of divisions that they would normally undergo in the presence of PDGF alone. While it appears that this continued functioning of the biological clock was able to bring cells to the brink of differentiation, it was not sufficient to cause a cessation of cell division or to induce differentiation. These results represent an extension of previous studies of this phenomenon, in that the measurement of time by O-2A progenitors had previously only been observed as the timed switching of cells from a program of self-renewal by division to a program of differentiation, in the presence of PDGF (Noble and Murray, 1984; Temple and Raff, 1986; Noble *et al.*, 1988; Raff *et al.*, 1988). From these studies it was not possible to determine whether the clock is part of the mechanisms that regulate cell division and differentiation or is separate from them. The demonstration that the measurement of time apparently occurs under conditions where division continues in the absence of differentiation allows us to examine this issue.

Predictions about the behavior of the clock under conditions in which cells divide but do not differentiate differ depending on whether it is a separate and independent mechanism. If the clock were an integral part of either the cell division or differentiation machinery, it would follow that the clock would be inoperative under the growth conditions we examined. In detail, if the clock worked by limiting the number of cell divisions, for example by the loss of an activity required for execution of the cell cycle, then conditions under which this limit is removed would prevent the measurement of time. Similarly, if the clock operated by inducing differentiation after a set period of time, conditions which prevent differentiation would destroy the cell's ability to measure time. Our observations appear to rule out both of these groups of potential mechanisms, as our

data suggest that the measurement of time is ongoing under conditions that induce continued proliferation in the absence of differentiation. Instead, we would suggest that our results are more consistent with the hypothesis that the measurement of time is performed by a separate cellular mechanism that can interact with the mechanisms controlling division and/or differentiation, yet is distinct from these mechanisms.

It should be noted that our experiments do not address the questions of whether cell division is required for the functioning of the clock or whether it is the number of cell divisions that are being counted. In order to examine these issues it would be necessary to analyze the ability of cells to measure elapsed time in the absence of cell division. Therefore, while our hypothesis states that the clock is separate from the cell division machinery (i.e., does not function by imposing an irrevocable limit on the number of cell divisions), it does not state that the clock does not require cell division to function.

Previous analysis of O-2A progenitors in single cell culture has led to the estimate that a maximum of eight rounds of cell division occur in response to stimulation by purified astrocytes (Temple and Raff, 1986), which appear to exert their mitogenic effects through PDGF (Noble *et al.*, 1988; Raff *et al.*, 1988; Richardson *et al.*, 1988). This estimate is at odds with our finding that growth for between 4 and 6 weeks in PDGF plus bFGF is required for O-2A progenitors to reach the point at which they are unresponsive to Ast/P (Figs. 1 and 2). O-2A progenitors grown in PDGF, or in PDGF plus bFGF, appear to have similar cell cycle times of approximately 20 hr (Noble *et al.*, 1988; Bögler *et al.*, 1990). At such a rate of division, at least 28 divisions (4 weeks; see Fig. 2) would be required to exhaust the measurement of time in the presence of PDGF plus bFGF.

One explanation for this difference between our observations and those of Temple and Raff (1986) would be that exposure to bFGF alters the clock by extending the period of time to be measured. This possibility has been raised in the context of a model for the clock whereby the measurement of time is ascribed to a reduction in the level of PDGF-α receptors, in a study where it was observed that bFGF could up-regulate the amount of PDGF-α receptor expressed by O-2A progenitors (McKinnon *et al.*, 1990). This possibility would not be inconsistent with our observation that a short exposure of embryonic optic nerve-derived O-2A progenitors to bFGF was associated with a slower production of oligodendrocytes, although the time of their first appearance was not changed (Fig. 3). However, other data from our study show that exposure to bFGF (in the presence of PDGF) for 1 week or longer leads to a reduction in the subsequent period of division in response to PDGF, suggesting that exposure to bFGF does not inevitably lead to an extension in the clock.

Another possible explanation for the discrepancy between our results and those of Temple and Raff (1986) is that the experimental conditions under which the measurement of time is examined can affect the result. When we analyzed individual O-2A progenitors as opposed to small populations, we observed that a shorter period of previous culture in PDGF plus bFGF resulted in the complete absence of division: for small population analysis 6 weeks was required, while for single cell analysis only 4 weeks sufficed (compare Figs. 1 and 2). This would suggest that any measure of the length of the PDGF responsive period derived from observations in single cell microculture may lead to an underestimate when compared to experiments using small populations. It is not clear whether these differences are due to interactions between O-2A progenitors, changes in the ratio of astrocytes to optic nerve cells, or other factors.

O-2A progenitors that differentiate under the control of the clock lose the ability to divide in response to PDGF before any overt phenotypic changes occur (Temple and Raff, 1986; Noble *et al.*, 1988; Raff *et al.*, 1988). Oligodendrocytes are however not truly postmitotic as they retain the ability to divide in response to bFGF for a period of time in culture (Eccleston and Silberberg, 1985; Saneto and De Vellis, 1985; Noble *et al.*, 1988; Bögler *et al.*, 1990; Mayer *et al.*, 1993). It has, therefore, appeared probable that a key event in triggering timed differentiation in O-2A progenitors is the selective loss of mitotic responsiveness to PDGF. This has seemed unlikely to be due to a loss of PDGF receptors, as at least 50% of newly formed oligodendrocytes express detectable levels of PDGF receptors (Hart *et al.*, 1989b). Furthermore, the PDGF receptors on immature oligodendrocytes are able to transmit signals: PDGF is capable of inducing a rise in intracellular calcium (Hart *et al.*, 1989a) as well as the expression of the proto-oncogenes c-fos and c-jun (Hart *et al.*, 1992). In addition, PDGF can transiently act as a survival factor for oligodendrocytes (Barres *et al.*, 1992) and elevate protein levels of the transcription factor SCIP/Tst-1/Oct-6 in immature oligodendrocytes (O. Bögler, A. Entwistle, R. Kuhn, G. Lemke, and M. Noble, unpublished observation). Our observation that O-2A progenitors that have become refractory to the mitogenic effect of PDGF after prolonged culture in PDGF plus bFGF, but continue to express easily detectable levels of PDGF receptor, is therefore in agreement with the work of others. The possibility that changes too subtle to be discerned with

either anti-PDGF receptor antibodies (this study) or radiolabeled PDGF (Hart *et al.*, 1989b) underlie timed differentiation cannot, however, be excluded at present.

Recent studies conducted on fibroblast populations suggest that the type of phenomenon we have observed is not restricted to O-2A progenitor cells. These experiments have been performed on fibroblast populations derived from H-2K^b-tsA58 transgenic mice (Jat *et al.*, 1991), which can be induced to express the immortalizing tsA58 mutant of SV40 large T antigen immediately after dissection by growth of cells in the presence of interferon at 33°C. Thus, these cells can be exposed to a stimulus that allows them to continue dividing beyond their normal limited mitotic life span from the beginning of their *in vitro* growth, in analogy with the exposure of O-2A progenitors to PDGF plus bFGF. Fibroblasts derived from H-2K^b-tsA58 transgenic mice are only conditionally immortal, in that switch to growth in interferon-free medium at 39.5°C turns off function of the large T antigen, again in analogy with the switch of O-2A progenitors from growth in PDGF plus bFGF to growth in Ast/P. It was shown previously that inactivation of a conditional immortalizing stimulus in long-term rodent fibroblast cultures was associated with the cessation of growth and an accumulation of cells in the G1 and G2/M phases of the cell cycle, suggesting that the removal of active large T antigen led to the recapitulation of senescence (Grove and Cristofalo, 1977; Jat and Sharp, 1989). The more recent studies on fibroblasts derived from H-2K^b-tsA58 transgenic mice have indicated that in these cells the normal mitotic life span is measured despite the presence of large T antigen. Thus, cells switched to nonpermissive conditions after various numbers of passages under immortalizing conditions have a shorter remaining mitotic life span than control cells, with the extent of remaining mitotic life span being related directly to the length of time the cells were first grown under immortalizing conditions. Moreover, cells switched after the normal mitotic life span has elapsed rapidly cease cell division and become senescent (Ikram *et al.*, in press). These results suggest that cells prevented from undergoing senescence by the presence of an immortalizing oncogene retain cellular memory of having passed the number of cell divisions after which they would have ordinarily senesced, implying that the mechanism that regulates the onset of senescence measures time in the presence of an oncogene. The timing mechanisms that regulate senescence and oligodendrocyte differentiation therefore appear to be at least superficially similar in that they remain active when not being able to regulate the onset of the processes they normally control. The extent to which such processes are indeed controlled by similar mechanisms will only be established when the molecular basis of timed differentiation is understood.

We suggest that the data we have presented establish that the biological clock of O-2A progenitor cells is an autonomous mechanism distinct from the mechanisms which allow cell division or promote cell-type-specific differentiation. An elucidation of the molecular nature of the clock may be facilitated by the ability to generate cells which are no longer able to measure time, for example by prolonged culture of O-2A progenitors in PDGF plus bFGF or rodent fibroblasts harboring a temperature-sensitive SV40 large T antigen. Such cells could be used as targets for attempts to reconstitute the measurement of time and so may lead to the identification of the molecules involved.

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EVIDENCE APPENDIX
EXHIBIT 15

Raff et al., "Platelet-derived Growth Factor from Astrocytes Drives the Clock that Times Oligodendrocyte Development In Culture," *Nature* 333:562-65 (1988)

Table 3 Anti-PDGF antibodies block response of O-2A progenitors to type-1 astrocytes and PDGF, but not to FGF

	Radiolabelled O-2A progenitors (%) No anti-PDGF	Plus anti-PDGF
ACM	63 + 4.2%	<1%
PDGF, 5 ng/ml	69 + 3.7%	<1%
FGF, 5 ng/ml	40 + 4.2%	44.5 + 2.5%

Cells were grown as in Table 1. Cultures received either the indicated mitogen, or mitogen plus 50 µg of rabbit anti-PDGF antiserum (purified Ig fraction, a kind gift of C. Heldin). When antibody was added, the ACM or non-conditioned medium with mitogen was preincubated with antibody for at least 1 h before addition to the cells. Cells were labelled with [^{3}H]-thymidine, immunolabelled, processed for autoradiography and scored as in Table 2.

the medium contained PDGF (data not shown). Thus, PDGF neither inhibited nor induced differentiation of O-2A progenitor cells into type-2 astrocytes, nor did it preclude differentiation of O-2A progenitor cells into oligodendrocytes. In these respects also, the effects of PDGF were identical to the effects of type-1 astrocytes.

To determine whether the effects of type-1 astrocytes were mediated by PDGF, we treated ACM, or medium containing PDGF or fibroblast growth factor (FGF) with affinity-purified anti-PDGF antibody. (As will be discussed elsewhere, FGF is also a mitogen for O-2A lineage cells, but does not mimic the effects of type-1 astrocytes on motility and differentiation.) These antibodies blocked the effects of ACM and PDGF, but did not block FGF-induced DNA synthesis in O-2A progenitors (Table 3), indicating that blocking was not due to toxic effects of the antibody.

Type-1 astrocytes also support the appropriately timed differentiation of embryonic O-2A progenitors grown *in vitro*[8]. As discussed in the accompanying paper[7], astrocyte-derived PDGF seems to play a key role in this effect and is by itself sufficient to promote the synchronous differentiation of clonally related and dividing progenitor cell families. Thus, in these respects also, PDGF completely mimics the effects of type-1 astrocytes.

The ability of a single molecule to replace type-1 astrocytes in modulating O-2A progenitor development *in vitro* suggests that these cells have a complex and constitutive behavioural phenotype, controlled by processes internal to the progenitors themselves. Progenitors stimulated to divide by an appropriate mitogen appear to be intrinsically migratory cells, with a bipolar morphology, which cease migration upon differentiating into multipolar oligodendrocytes; this differentiation seems to be controlled, at least in part, by internal clocks which may function by counting cell divisions. This programme does not, however, include astrocyte differentiation, which requires a separate inducing factor[10].

The observations that PDGF induced DNA synthesis at picomolar concentrations, the identical effects of PDGF and type-1 astrocytes on the division, differentiation and motility of O-2A progenitors and the ability of anti-PDGF antibodies to block the activity of astrocyte-conditioned medium all indicate that the astrocyte activity is a PDGF-like substance. In addition, messenger RNA for PDGF has been identified in purified astrocytes and these cells have been found to secrete PDGF *in vitro*[17] (W. Richardson, N. Pringle, M. Moseley, B. Westermark, & M. Dubois-Dalcq, manuscript submitted). Together, our studies indicate that PDGF may play an important role in gliogenesis in the CNS.

The ability of PDGF to promote division and migration of O-2A progenitor cells may be of particular interest in light of observations that PDGF can act as a chemotactic agent[11]. During embryogenesis, O-2A progenitors appear to populate the optic nerve by migrating from a germinal zone in or near the optic chiasm along the nerve towards the eye[5]. This directional migration could be due to movement along a gradient of a chemotactic substance, such as PDGF. In the adult animal, the ability of cells in a lesion site to secrete compounds which promote O-2A progenitor migration and division could be of value in the repair of demyelinating damage in the CNS. The controlled application of PDGF, or other chemotactic mitogens, might enhance these repair processes.

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Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture

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The various cell types in a multicellular animal differentiate on a predictable schedule but the mechanisms responsible for timing cell differentiation are largely unknown. We have studied a population of bipotential glial (O-2A) progenitor cells in the developing rat optic nerve[1] that gives rise to oligodendrocytes beginning at birth and to type-2 astrocytes[2] beginning in the second postnatal week[3]. Whereas, *in vivo*, these O-2A progenitor cells proliferate and give rise to postmitotic oligodendrocytes over several weeks[4,5], in serum-free (or low-serum) culture they stop dividing prematurely and differentiate into oligodendrocytes within two or three days[1,6,7]. The normal timing of oligodendrocyte development can be restored if embryonic optic-nerve cells are cultured in medium conditioned by type-1 astrocytes[8], the first glial cells to differentiate in the nerve[9]: In this case the progenitor cells continue to proliferate, the first oligodendrocytes appear on the equivalent of the day of birth, and new oligodendrocytes continue to develop over several weeks, just as *in vivo*[7]. Here we show that platelet-derived growth factor (PDGF) can replace type-1-astrocyte-conditioned medium in restoring the normal timing of oligodendrocyte differentiation *in vitro* and that anti-PDGF antibodies inhibit this property of the appropriately conditioned medium. We also show that PDGF is present in the developing optic nerve. These findings suggest that type-1-astrocyte-derived PDGF drives the clock that times oligodendrocyte development.

Table 1 Effect of PDGF on O-2A progenitor-cell proliferation and differentiation into oligodendrocytes *in vitro*

Age of optic nerve	PDGF (ng ml^{-1})	Days in culture	Number of O-2A progenitor cells	Number of oligo-dendrocytes
E17	0	2	8 ± 5	15 ± 7
E17	0	3	5 ± 3	19 ± 8
E17	2	2	51 ± 8	0
E17	2	3	117 ± 21	0
E17	2	4	214 ± 38	4 ± 2
E18	0	2	8 ± 3	55 ± 7
E18	2	2	135 ± 17	0
E18	2	3	317 ± 45	10 ± 4
E19	0	1	14 ± 2	70 ± 4
E19	2	1	120 ± 9	0
E19	2	2	199 ± 28	2 ± 1
E18	2	2	148 ± 13	0
E18	2	3	287 ± 23	12 ± 6
E18	10	2	133 ± 11	0
E18	10	3	278 ± 37	8 ± 4

Optic nerves from embryonic S/D rats were dissociated into single cells and cultured on poly-D-lysine (PDL)-coated glass coverslips (about 30,000 cells per coverslip) in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose, insulin, transferrin, bovine serum albumin, progesterone, putrescine, thyroxine, tri-iodothyronine and 0.5% FCS as described[7]. Purified human PDGF (R and D Systems, Inc.) was added at the start of the culture. After 1–4 days, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (*p*H 7.5) for 5 min at room temperature and stained successively with monoclonal anti-galactocerebroside (GC) antibody[19] (ascites fluid diluted 1:1000), fluorescein-coupled goat anti-mouse IgG3 (G anti-IgG3-Fl, Nordic, diluted 1:100), A2B5 monoclonal antibody[20] (ascites fluid diluted 1:100) and finally rhodamine-coupled goat anti-mouse Ig (G anti-MIg-Rd, Cappel, diluted 1:100); the cells were then post-fixed in acid-alcohol, mounted in glycerol and examined in a Zeiss Universal fluorescence microscope, as described[7]. O-2A progenitor cells were identified by their antigenic phenotype (A2B5$^+$, GC$^-$)[1,15] and characteristic process-bearing morphology[15], while oligodendrocytes were identified as GC$^+$ process-bearing cells[11]. The total numbers of these cells were counted on each coverslip and the results are expressed as means ± s.d. of at least three experiments. The concentration of PDGF required for half-maximal stimulation of O-2A progenitor-cell proliferation was ~0.5 ng ml^{-1} (ref. 9).

We were led to study the effect of PDGF on the timing of oligodendrocyte development *in vitro* by recent evidence that PDGF is a potent mitogen for O-2A progenitor cells in culture[9,10] and that cultured type-1 astrocytes stimulate O-2A progenitor-cell proliferation[8] by secreting PDGF[9]. In the present study, optic nerve cells from embryonic day 17 (E17) Sprague–Dawley (S/D) rats were cultured in medium containing 0.5% fetal calf serum (FCS). As previously reported[7], within two days most of the O-2A progenitor cells in such cultures stopped dividing and differentiated into oligodendrocytes, which were identified by the binding of antibody against galactocerebroside (GC)[11] (Table 1). When human PDGF (R and D Systems, Inc., Minneapolis) was added, however, the O-2A progenitor cells continued to proliferate, doubling in number approximately every day, and the first oligodendrocytes developed after four days, equivalent to the time of birth (Table 1). The same result was obtained if the cells were cultured in type-1-astrocyte-conditioned medium (ACM), as reported previously[7] (data not shown). When E18 or E19 optic nerve cells were cultured in PDGF, the first oligodendrocytes developed after three and two days, respectively, again equivalent to the day of birth (Table 1). The same results were obtained with a fivefold higher concentration of PDGF (Table 1), with human PDGF obtained from Raines and Ross[12], and with porcine PDGF obtained either from R and D Systems or from Stroobant and Waterfield[13] (data not shown).

These results indicate that PDGF can mimic the effects of ACM in restoring in culture the normal timing of oligodendrocyte development observed *in vivo*, raising the possibility that PDGF is the factor in ACM responsible for this activity. To test this possibility, E17 optic nerve cells were cultured in ACM together with an IgG fraction of a goat anti-PDGF antiserum. As shown in Table 2, these antibodies completely blocked the ability of ACM both to stimulate O-2A progenitor-cell proliferation (as reported previously[9]) and to restore the normal *in vivo* timing of oligodendrocyte development in culture (Table 2). The same result was obtained using an IgG fraction of a rabbit antiserum[14] obtained from C.-H. Heldin (data not shown). IgG fractions of goat and rabbit antisera against mouse immunoglobulin, used at the same or tenfold higher concentration, had no such effect (data not shown). The addition of exogenous PDGF together with the anti-PDGF antibodies completely overcame the inhibitory activity of the antibodies (Table 2).

Table 2 Effect of anti-PDGF antibodies on the ability of conditioned medium (ACM or ONCM) to restore the *in vivo* timing of oligodendrocyte development in cultures of E17 optic nerve cells

Conditioned medium	Anti-PDGF antibodies (90 μg ml^{-1})	PDGF (ng ml^{-1})	Number of O-2A progenitor cells	Number of oligo-dendrocytes
none	–	0	12 ± 5	25 ± 11
none	–	2	71 ± 9	0
ACM	–	0	75 ± 13	0
ACM	+	0	15 ± 7	24 ± 8
ACM	+	15	82 ± 18	0
ONCM	–	0	64 ± 8	0
ONCM	+	0	8 ± 2	31 ± 5

Cells from E17 optic nerves were prepared, cultured for 2 days and stained as in Table 1. Purified type-1 astrocytes from newborn rat cerebral cortex were prepared as described[21]; after growing for several weeks in DMEM supplemented with 10% FCS, the astrocytes were grown in the defined medium (with 0.5% FCS) described in Table 1 for 2 days and the medium was collected as ACM. Newborn optic nerve cells (5×10^5 in 2 ml in PDL-coated 3.5 mm Nunc tissue-culture dishes) were cultured as described[1]; after 1 day in DMEM containing 10% FCS, the cultures were switched to defined medium containing 0.5% FCS, which was collected after 1 day as ONCM. The conditioned media were tested on E17 optic-nerve cultures to find the highest dilution that would still restore the normal timing of oligodendrocyte differentiation, and this concentration (which varied from undiluted to diluted 1:10) was used in these experiments. The medium was changed after 24 h, and fresh conditioned medium, anti-PDGF antibody, and human PDGF (R and D Systems, Inc.) were added. The goat anti-human PDGF antibodies (an IgG fraction prepared by ion-exchange chromatography) were purchased from Collaborative Research Inc. (Bedford, Massachusetts); 50 μg ml^{-1} of the antibody completely neutralized the mitogenic activity of 5 ng ml^{-1} of human PDGF for O-2A progenitor cells (data not shown), and for NIH 3T3 cells (according to the Collaborative Research specification sheet). The results are expressed as means ± s.d. of three separate experiments, except for the results with ONCM where they are triplicates of a single experiment.

The ACM used in the present and previous studies[7-9] was derived from cultures of type-1 astrocytes purified from neonatal-rat cerebral cortex. To determine whether type-1 astrocytes in optic nerve cell cultures also secrete PDGF, we cultured E17 optic nerve cells in medium conditioned over a high density culture of newborn optic nerve cells; almost 60% of newborn optic nerve cells are type-1 astrocytes[3]. As shown in Table 2, such optic nerve conditioned medium (ONCM) kept O-2A progenitor cells dividing and prevented these cells from prematurely differentiating into oligodendrocytes; this activity was

completely inhibited by anti-PDGF antibodies. We have previously provided evidence, however, that the endogenous type-1 astrocytes in E17 optic nerve cultures are too few in number and/or are unable to recover quickly enough from the dissociation procedure to produce sufficient mitogen to keep the progenitor cells dividing and to prevent their premature differentiation[7].

To determine whether PDGF is made in the developing optic nerve, we tested an extract of three-week-old rat optic nerve for its ability to stimulate O-2A progenitor cells in culture to incorporate bromodeoxyuridine (BrdU) into DNA before and after the extract was treated with anti-PDGF antibodies. As shown in Table 3, the extract stimulated progenitor cells to incorporate

Table 3 Stimulation of BrdU incorporation in O-2A progenitor cells by optic nerve extract in culture: the effect of anti-PDGF antibodies

Additives	% O-2A progenitor cells labelled with BrdU
None	3±1
PDGF (1ng ml^{-1})	64±4
Optic nerve extract	50±2
Optic nerve extract treated with anti-PDGF antibodies	14±6

Cells from newborn optic nerves were cultured as in Table 1, except that they were maintained without FCS at 5,000 cells per culture. Bromodeoxyuridine (BrdU, 10 μM; Boehringer) was added after 11.5 h, and the cultures were fixed after 48 h and stained with A2B5 antibody, followed by G anti-MIg-F1 (Cappel, 1:100); they were then treated successively with 2 N HCl (to denature the nuclear DNA[22]), 0.1 M $Na_2B_4O_7$, pH 8.5 (each for 10 min at room temperature), monoclonal anti-BrdU antibody[23] (culture supernatant diluted 1:5) and finally with G anti-MIgRd. Optic-nerve extract was prepared from three week-old rats as described[14] and used at 220 μg total protein ml^{-1}. For treatment with anti-PDGF antibodies, the extract (264 μg total protein) was incubated with the IgG fraction of goat anti-human PDGF antiserum (135 μg in 0.7 ml of DMEM; Collaborative Research) for 4 h at 4 °C with continuous rotation; protein A-Sepharose (50 μl of swollen gel, Pharmacia) was added and the mixture was incubated for a further 12 h at 4 °C with continuous rotation and then centrifuged for 1 min in an MSE Micro Centaur. Incubating the extract in normal goat serum and then protein A-Sepharose had no effect on the extract's activity; the extract used in the experiment shown was treated in this way. Anti-PDGF antibodies inhibited the activity of the extract to the same extent when they were added directly (85 ng ml^{-1}) to the cultures (data not shown). The results are expressed as means ± s.e.m. of triplicate cultures.

BrdU, and more than 70% of this mitogenic activity was removed by anti-PDGF antibodies. While this suggests that the major mitogen for O-2A progenitor cells in the optic nerve at this age is PDGF, the finding that not all of the mitogenic activity in the extract was removed by the antibodies, or neutralized when progenitor cells were cultured with the extract in the presence of an excess of anti-PDGF antibody (data not shown), suggests that another mitogen(s) is also present.

Our present and previous results provide compelling evidence that PDGF, secreted by type-1 astrocytes, regulates both the proliferation and the timing of differentiation of O-2A progenitor cells *in vitro*. To summarize the evidence: (1) PDGF stimulates the proliferation of O-2A progenitor cells and prevents them from differentiating prematurely into oligodendrocytes in culture (refs 9, 10, and this study); (2) cultures of type-1 astrocytes purified from cerebral cortex make both PDGF and messenger RNA encoding PDGF A chains[8]; (3) when ACM is fractionated by gel filtration, the mitogenic activity for O-2A progenitor cells is found in the same fractions as radiolabelled PDGF[8]; (4) anti-PDGF antibodies inhibit the ability of ACM and ONCM to stimulate O-2A progenitor cell proliferation *in vitro* (ref. 9, 10 and this study) and to restore the normal *in vivo* timing to oligodendrocyte differentiation (this study).

Table 4 Clonal analysis of oligodendrocyte differentiation in optic nerve cell cultures from 7-day-old rats stimulated by PDGF

Clones	Number of cell divisions: 0	1	2	3	4
a,b,c	1P	2o[l]			
d,e,f	1P	2P	4o[l]		
g	1P	2P	4P	8o[l]	
h	1P	2P	3P,1M	6o[l]	
i	1P	2P	4P	8P	16o[l]
j	1P	2P	4P	5P,1o[l],1M	11o[l]

Cells from post-natal day 7 (P7) S/D rats were prepared and studied by time-lapse microcinematography as described[16], except that the cells were maintained in human PDGF (10 ng ml^{-1}) instead of ACM. Proliferation and differentiation of cells in microscopic fields containing 2 to 4 O-2A progenitor cells were followed for one week. The O-2A progenitor cells were identified by their characteristic bipolar morphology[13,16] and migratory behaviour[16], while oligodendrocytes were identified by their multipolar morphology[10,13,16] and lack of motility[16]. As shown in the table, oligodendrocyte differentiation occurred after one to four cell divisions in the ten clones studied, although in every experiment there were still dividing O-2A progenitor cells (belonging to other clones) present in the field at the end of filming. P, O-2A progenitor cells; ol, oligodendrocytes; M, cells that migrated out of the microscopic field and could no longer be followed.

Previous studies have suggested that ACM regulates the timing of oligodendrocyte development *in vitro* by keeping O-2A progenitor cells dividing until an intrinsic clock in the progenitor cell initiates the process that leads to oligodendrocyte differentiation[7,13]. The results of these studies were consistent with the possibility that the clock operates by setting a maximum number of divisions a progenitor cell and its progeny can undergo before differentiating. To test this possibility further we cultured optic nerve cells from seven-day-old rats in the presence of PDGF and followed the fate of individual O-2A progenitor cells and their progeny by time-lapse microcinematography. As described previously, O-2A progenitor cells and oligodendrocytes could be easily recognized as motile bipolar cells and immotile multipolar cells, respectively[16]. In nine of the ten clones studied, all of the descendants of a single progenitor cell differentiated together into non-dividing oligodendrocytes after the same number of cell divisions (Table 4); in the other clone (clone j in Table 4), the progenitor cells differentiated within one cell division of one another. These findings are consistent with previous single-cell experiments and the 'mitotic clock' hypothesis[13] but do not exclude other timing mechanisms.

Whatever the timing mechanism, it is clear that oligodendrocyte differentiation is associated with withdrawal from the cell cycle[4,5,8]. The relationship between the two processes, however, is uncertain. The clock in the progenitor cell might primarily control the onset of oligodendrocyte differentiation, with the cessation of proliferation following as a consequence. Alternatively, the clock might primarily control the onset of unresponsiveness to PDGF, with oligodendrocyte differentiation following as a consequence of withdrawal from the cell cycle. We favour the second possibility as it would most simply explain why O-2A progenitor cells differentiate prematurely when deprived of PDGF (see Table 2).

Whereas oligodendrocyte differentiation seems to be the constitutive pathway of O-2A progenitor cell development, which is automatically triggered when a progenitor cell is deprived of signals from other cells[17] or when the instrinsic timer reaches the appropriate point[7,15], type-2 astrocyte differentiation seems to be induced by a protein signal that greatly increases in concentration in the optic nerve after the first postnatal week[18]. While the mechanisms that control the timing and direction of O-2A progenitor cell differentiation *in vitro* are beginning to emerge, it remains to show that the same mechanisms operate *in vivo*.

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The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria

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Fc receptors on phagocytic cells in the blood mediate binding and clearance of immune complexes, phagocytosis of antibody-opsonized microorganisms, and potently trigger effector functions, including superoxide anion production and antibody-dependent cellular cytotoxicity. The Fc receptor type III (FcγR III, CD16), present in 135,000 sites per cell 1 on neutrophils and accounting for most of FcR in blood, unexpectedly has a phosphatidylinositol glycan (PIG) membrane anchor. Deficiency of FcγR III is observed in paroxysmal nocturnal haemoglobinuria (PNH), an acquired abnormality of haematopoietic cells[2] affecting PIG tail biosynthesis or attachment[3], and is probably responsible for circulating immune complexes[4] and susceptibility to bacterial infections associated with this disease[5]. Although a growing number of eukaryotic cell-surface proteins with PIG-tails are being described[6,7], none has thus far been implicated in receptor-mediated endocytosis or in triggering of cell-mediated killing. Our findings on the FcγR III raise the question of how a PIG-tailed protein important in immune complex clearance *in vivo*[8,9] and in antibody-dependent killing[10] mediates ligand internalization and cytotoxicity. Together with our results, previous functional studies on FcγR III and FcγR II[11,12] suggest that these two receptors may cooperate and that the type of membrane anchor is an important mechanism whereby the functional capacity of surface receptors can be regulated.

Three different types of FcγR have been distinguished in humans using monoclonal antibodies[13] (mAb). FcγR III (CD16) of relative molecular mass (M_r) 50–70,000 (50–70K) is found on neutrophils, large granular lymphocytes, and macrophages, but not on monocytes. FcγR III was first identified with a mAb (3G8) that blocked immune complex binding to neutrophils[1] and subsequently with other mAb of the CD16 cluster[14]. FcγR II (CDw32) is a 40K receptor on neutrophils, monocytes, eosinophils, platelets and B cells[13]. FcγR I is a 72K protein and is found on monocytes[12]. FcγR III and FcγR II have low affinity for monomeric IgG and thus preferentially bind immune complexes by multiple receptor–ligand interactions, whereas FcγR I is sufficiently high affinity to bind monomeric IgG.

Our first evidence that FcγR III is anchored by PIG came from studies on leukocytes from patients with paroxysmal nocturnal haemoglobinuria (PNH). PNH is an acquired defect of haematopoietic precursor cells in either the biosynthesis or the attachment of the PIG tail and may affect clonal progeny in the erythroid, monocytic, granulocytic, and thrombocytic lineages[2,15,16]. Previous studies on erythrocytes and leukocytes from PNH patients have demonstrated a selective deficiency of PIG-tailed proteins, including decay accelerating factor (DAF), acetylcholinesterase, alkaline phosphatase and the PIG-anchored form of lymphocyte function-associated antigen 3 (LFA-3), (refs 3, 6 and 7). The deficiency of DAF accounts for susceptibility of erythrocytes to complement-mediated lysis in PNH. However none of these previously identified deficiencies can explain the occurrence of circulating immune complexes[4] and the 20% and 50% of mortalities caused by bacterial infections and thrombosis respectively[5].

Quantitation of FcγR III expression using immunofluorescence flow cytometry show that it is markedly deficient on PNH neutrophils (Fig. 1*a*). This deficiency was found in all six patients studied (D.E., S.B., J.M., J.E., B.I., C.G.) and results with five different CD16 (FcγR III) mAb were identical. Some patients such as J.E. (Fig. 1*a*, curve 3) showed normal as well as deficient granulocyte clones. Patients showed consistent variation in the extent of deficiency in the abnormal clone. The amount of FcγR III expression on affected cells ranged from 2% (patient D.E.) to 19% (patient J.E.) averaging 7% of normal, perhaps reflecting the degree of penetrance of the acquired defect in PNH. In all cases, deficiency of FcγRIII paralleled deficiency of DAF. In contrast, deficient neutrophils expressed normal levels of HLA-A,B, LFA-1, Mac-1 and FcγR II (CDw32) (Fig. 1*a*). PNH monocytes showed normal expression of FcγR I and II, although they were deficient in DAF (Fig. 1*b*). These results suggested that the neutrophil FcγR III has a PIG tail, whereas the FcγR I and FcγR II do not.

PIG-anchored proteins can be specifically cleaved from cell surfaces with phosphatidylinositol-specific phospholipase C[3,6,7] (PIPLC). We therefore investigated the susceptibility of Fc receptors to PIPLC (Table 1). PIPLC released 75–84% of the cell surface FcγR III and DAF from healthy granulocytes, while FcγR II, HLA-A,B and LFA-1 were unaffected. On monocytes, PIPLC released 84% of DAF whereas it had no effect on FcγR II, FcγR I, HLA-A,B and LFA-1. Results with PIPLC prepared from *S. aureus* and *B. thuringiensis* were identical and show that FcγR III on neutrophils, but not FcγR II on the same cells or FcγR I and II on monocytes, have PIG anchors. Lack of a PIG anchor on FcγR II is consistent with the prediction (from cDNA sequence[17]) that it possesses a transmembrane domain and a 76 residue hydrophilic cytoplasmic tail.

** TOTAL PAGE. 06 **